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Characterization of Herc5: the major ligase for ISG15, an antiviral ubiquitin-like protein

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Characterization of Herc5: the major ligase for ISG15, an antiviral ubiquitin-like protein

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Human ISG15 is a 17 kDa ubiquitin-like protein (Ubl) that is induced by type I interferons (interferons α and β) and plays a role in antiviral responses. ISG15 is conjugated via its C-terminus to more than 150 cellular proteins, and like ubiquitin, an E1-E2-E3 enzymatic cascade is required for conjugation. Ube1L and UbcH8 were previously identified as the E1 and E2 enzymes for this pathway. My experiments identified Herc5, a HECT domain E3, as the major ligase for ISG15. Like ISG15, Ube1L, and UbcH8, expression of Herc5 is transcriptionally induced by type I interferons. siRNAs against Herc5 abrogated ISG15 conjugation to the vast majority of target proteins in interferon-treated cells. Wild type Herc5, but not the catalytically inactive C994A mutant, supported conjugation of ISG15 in non-interferon-treated cells co-

transfected with Ube1L, UbcH8 and ISG15. IQGAP1, a scaffold protein, was identified as another essential component of the ISG15 system. IQGAP1 was discovered to interact with Herc5, and this interaction was mediated by the C-terminal domain of IQGAP1 and the N-terminal RCC1-like repeats of Herc5. IQGAP1 was required for auto-conjugation of ISG15 to Herc5, and I propose a model where IQGAP1 functions, at least in part, by relieving an auto-inhibitory conformation of Herc5.

Thus, I have identified two factors that are critical for ISG15 conjugation and my discoveries have increased our understanding of the ISG15 pathway. Identification and characterization of the conjugation apparatus will aid in establishing an *in vitro* biochemical system for ISG15 conjugation, which in turn, will be important to decipher the biological function of ISG15 modification.

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CHAPTER 1: INTRODUCTION

1.1 Prelude

Ubiquitin was first discovered in the bovine thymus in 1974 as an 8.5 kDa polypeptide that had lymphocyte differentiating properties (44). It was thought that this protein was expressed across all kingdoms of life, including prokaryotes, and hence was given the name ubiquitous immunopoeitic polypeptide (UBIP). Later it was discovered that a contaminating endotoxin was responsible for stimulating the lymphocytes and that this polypeptide was restricted to eukaryotes.

A few years and several pioneering experiments later, the role of ubiquitin in proteolysis was discovered. It was observed that cell free rabbit reticulocyte lysates degraded abnormal haemoglobin in an ATP-dependent manner (152). This was the first time that a cell free proteolytic system that did not involve lysosomes had been isolated (28). Reticulocytes were a useful source to purify and characterize many enzymes involved in ubiquitin-proteasome biochemistry (15, 56), probably because of the large amount of protein degradation that occurs in reticulocytes as they differentiate to mature red blood cells. Another important milestone in ubiquitin research was the discovery that ubiquitin was covalently linked to other proteins, as first described for histones H2A and H2B (43, 73). These discoveries ultimately led to a new paradigm where, in an ATP-dependent manner, substrates are tagged with ubiquitin to mark them for degradation by the proteasome.

Today we know that ubiquitination is involved in virtually all major cellular processes, including the cell cycle and cell division, cell growth and differentiation, the activation and silencing of transcription, apoptosis, immune and inflammatory responses, signal transduction, receptor-mediated endocytosis and the sorting of proteins in the cell. We also know that ubiquitination can take several forms and that it does not always serve as a degradation signal. It was due to the huge impact of ubiquitin in many different fields of biology that the 2005 Nobel Prize in Chemistry was awarded to Aaron Ciechanover, Avram Hershko and Irwin Rose, three scientists who played a major role in identifying the enzymes and deciphering the biochemistry involved in ubiquitination.

A major advance in the ubiquitin field was the discovery of ubiquitin-like proteins (Ubls), with ISG15 being the first Ubl to be discovered in 1979 (31). Ubls share sequence and structural similarity with ubiquitin, and are also conjugated to substrates via their C-terminal glycine residues. Most Ubls do not target proteins to the proteasome, but have various non-proteolytic functions, such as changing the localization, activity or binding partners of a substrate (94). The Ubls have greatly contributed to our understanding of the Ub/Ubl conjugation machinery. For example, the Nedd8 activating enzyme was the first such enzyme to be crystallized and this gave us general insights into the details of ubiquitin/Ubl activation (177).

The main goal of my doctoral research was to understand the mechanism of conjugation of ISG15, a ubiquitin-like protein involved in the innate immune response. When I started the project, the E1 and E2 enzymes for ISG15 had been discovered. I identified Herc5, a HECT E3, as the major ligase for this pathway. Further, I showed that

the scaffold protein IQGAP1 is an important component of the ISG15 conjugation machinery, and that IQGAP1 interacts with Herc5. My findings have enhanced our knowledge of the enzymes involved in ISG15 conjugation, which is a prerequisite for understanding the biological role of ISG15.

1.2 A brief overview of the ubiquitin-proteasome system

Conjugation of ubiquitin to substrates requires the sequential action of a set of three enzymes, the E1, E2 and E3 enzymes (149). The E1 or ubiquitin activating enzyme has binding sites for ATP and ubiquitin and it catalyzes the formation of a ubiquitin adenylate intermediate which activates the C-terminus of ubiquitin. A high-energy thioester bond is then formed between the E1 and ubiquitin by the nucleophilic attack of an E1 active-site thiolate on the activated ubiquitin C-terminus, with release of pyrophosphate. E1 also has a binding site for E2 enzymes, and ubiquitin is subsequently transferred via a trans-thiolation reaction to the active-site cysteine residue of E2 ubiquitin conjugating enzymes. E3 ubiquitin ligases facilitate the transfer of ubiquitin from the E2 onto an ε-amino group of a substrate lysine, forming an isopeptide bond. Ubiquitin is thus sequentially transferred via the E1-E2-E3 cascade onto the substrate. Most organisms have a single E1, a dozen or so E2s and several hundred E3s. Consistent with their role in mediating substrate interactions, E3s are the most abundant class of ubiquitinating enzymes.

E3s can be broadly classified into two classes: the RING (Really Interesting New Gene) type E3s (113) and the HECT (Homology to E6-AP C-terminus) type E3s (70). These two classes differ in the manner in which they catalyze substrate ubiquitination. The HECT E3s participate directly in the chemistry of ubiquitination, forming a thioester intermediate with ubiquitin before it is transferred onto an ε-amino group of a substrate lysine. This group of E3s is described in the next section below. RING E3s, on the other hand, act as scaffold proteins that bring the E2 in proximity to the substrate, thus facilitating the transfer of ubiquitin directly from the E2 to the substrate. The RING domain, which typically consists of seven cysteine residues and one histidine residue that intercalate a Zn atom, mediates interaction with the E2s (22). The RING E3s occur either as single polypeptides (e.g., Cbl, Mdm2) or as multi-protein complexes called Cullin-RING ligases (CRLs; e.g., SCF ligases and the APC) (149). SCF ligases are a class of well characterized CRLs that are composed of Rbx1/Roc1 (RING-finger protein), Skp1 (an adaptor protein), Cul1 (scaffold protein) and an F-box protein to mediate substrate interaction (147). The globular C-terminus of Cullins interacts with Rbx1/Roc1, which in turn binds E2s, whereas the N-terminus recruits substrates via Skp1 and other adaptor proteins. The variable component of SCFs is the F-box protein that binds Skp1 via its F-box motif and recruits substrates (5). Approximately 70 different F-box proteins have been identified in the human genome, and they contain various protein-protein interaction motifs such as the WD40 repeats and leucine rich repeats (77). Besides the F-box proteins, BTB (broad complex, tramtrack, bric-a-brac) domain proteins, and SOCS/BC (suppressor of cytokine signaling/elongin-BC) box proteins can also associate with

Cullins to recruit substrates (84, 187). Further, in higher eukaryotes, there are as many as seven cullin genes (Cul1-7) which can associate with different adaptor proteins, giving rise to hundreds of different SCF-like complexes. Thus, the basic Cullin-Rbx1-adaptor architecture is preserved, but owing to the diversity of subunits that can combine to assemble this core catalytic complex, the CRLs can target a variety of substrates for ubiquitin mediated degradation.

Another important class of enzymes involved in ubiquitin chemistry is the deubiquitinating enzymes (DUBs). It is estimated that the human genome encodes about 90 DUBs, regulating various physiological processes (66). A function of DUBs is to hydrolyze the isopeptide linkage between the C-terminus of ubiquitin and the substrate lysine, thus reversing ubiquitination (4). This also generates free ubiquitin monomers that can be recycled before the substrate is degraded by the proteasome. Another important biochemical reaction of DUBs is to process ubiquitin precursors into active forms. In cells, ubiquitin and most Ubls are produced as a fusion to either ribosomal proteins or to other ubiquitin molecules with additional residues extending beyond the last ubiquitin monomer (32). These extra amino acids are cleaved by DUBs to generate the 76 amino-acid active form of the protein with a C-terminal glycine residue.

Ubiquitin has seven lysine residues (K6, K11, K27, K29, K33, K48 and K63), each of which were shown *in vivo* to form iso-peptide bonds with the C-terminal glycine of other ubiquitin molecules (145). The type of linkage used to generate the resulting poly-ubiquitin chains determines, to some degree, the fate of the modified substrate. For example, a minimum of four K48-linked ubiquitin molecules are sufficient to direct a

substrate to the proteasome (174). K63 chains are involved in cellular processes such as DNA repair, receptor endocytosis and NF-κB activation (37, 60, 85). While K63 chains can have non-proteolytic functions, there is also evidence that K63 chains can target proteins to the proteasome. The biological relevance of other chain types is not yet known. Also, it is not clear what determines chain type specificity. In some cases, E2s are responsible for the chain type. An example is the E2 heterodimer Ubc13-Mms2, which catalyzes K63 chains (121). However, in our lab we observed two distinct chain types with the same E2, UbcH7, functioning with two separate HECT ligases: K48 chains with E6AP and K63 chains with Rsp5 (91). Kirkpatrick, *et al*, used quantitative mass spectrometry to show that the anaphase promoting complex (APC), a type of CRL, adds multiple mono-ubiquitin molecules on cylin B1, following which it catalyzes heterogeneous polyubiquitin chains (K63, K11 and K48) (98). More recently, the RING E3 Mdm2 was shown to catalyze a heterogeneous polyubiquitin chain that contains all seven possible linkages (95).

K48 linked polyubiquitin chains direct substrates to the 26S proteasome, which is a multi-subunit chambered protease. It is made up of a 20S core particle, and a 19S regulatory particle also known as the lid. The core particle is a barrel-shaped cylinder consisting of a stack of four rings: two identical outer α rings and two identical inner β-rings (150). Although the α and β rings are made up of similar seven-membered subunits, the protease activity resides within the β-ring only. The α-subunits mainly control the entry of substrates into and exit of products out of the interior compartments. The main

functions ascribed to the regulatory particle are recognition of the K48 chains, gating of the proteasome pore, and unfolding proteins before they enter the 20S subunit (150). Rpn10/S5A, a subunit of the 19S complex binds polyubiquitin chains and is thought to recruit K48-linked substrates to the proteasome (26). However, an Rpn10 deletion in yeast causes only a mild defect, indicating that other ubiquitin binding proteins must be involved. Opening the orifice at one end of the α-ring, and unfolding substrates are a prerequisite to accommodate proteins into this narrow barrel-shaped proteasome. These processes require energy, and six different ATPase subunits are found at the base of the 19S subunit. Rpn11/ POH1 is a Zn^{2+} dependent metalloprotease that is an essential subunit of the 19S complex (3). It removes intact ubiquitin subunits just before the unfolded substrate translocates into the pore of the 20S subunit, and this step is essential to enhance the rate of proteolysis.

1.3 HECT E3s:

The defining member of the HECT family of proteins is E6AP (E6-associated protein). It was identified as the cellular factor that associated with the E6 protein of human papillomaviruses 16 and 18 (HPV16 and HPV18) to target p53 for proteasomal degradation (71, 163). High risk HPVs (e.g., HPV16, 18, 33 and 39), are the leading cause of cervical cancer because the viral oncogenes, E6 and E7, mediate the degradation of p53 and the Retinoblastoma protein (Rb), respectively (65). Besides its role in cervical cancer, mutations in UBE3A, the gene encoding E6AP, are the cause of Angelman syndrome, a severe neurological disorder (16).

Sequence analysis revealed that the C-terminal 350 amino acids of E6AP were similar to the C-termini of other proteins, and this domain was named the HECT domain (69). HECT proteins have been identified in all eukaryotes and they vary in length from 90 kDa to over 500 kDa. While the HECT domain mediates catalysis, the N-termini of these proteins are involved in substrate recognition. For example, the yeast HECT E3 Rsp5, contains three WW domains upstream of the HECT domain, and at least two of these mediate interactions with substrates. Also, the E6-binding region of E6AP is a 17 amino acid peptide, centred approximately 100 residues upstream of the HECT domain. The biochemical activities of the HECT domain can be divided into 4 steps: (i) interaction with the E2, (ii) trans-thiolation reaction where ubiquitin is transferred from the E2 to the HECT active site cysteine, (iii) transfer of ubiquitin onto an ε-amino group of a lysine residue on the substrate, (iv) repeat of the activation steps and transfer of another molecule of ubiquitin onto one of seven lysine residues of a previously conjugated ubiquitin molecule (68). Structural and functional analyses have revealed residues important for the first three steps of the reaction. Ongoing research in our lab suggests that the C-lobe of the HECT domain (described below) is an important determinant for polyubiquitin chain type.

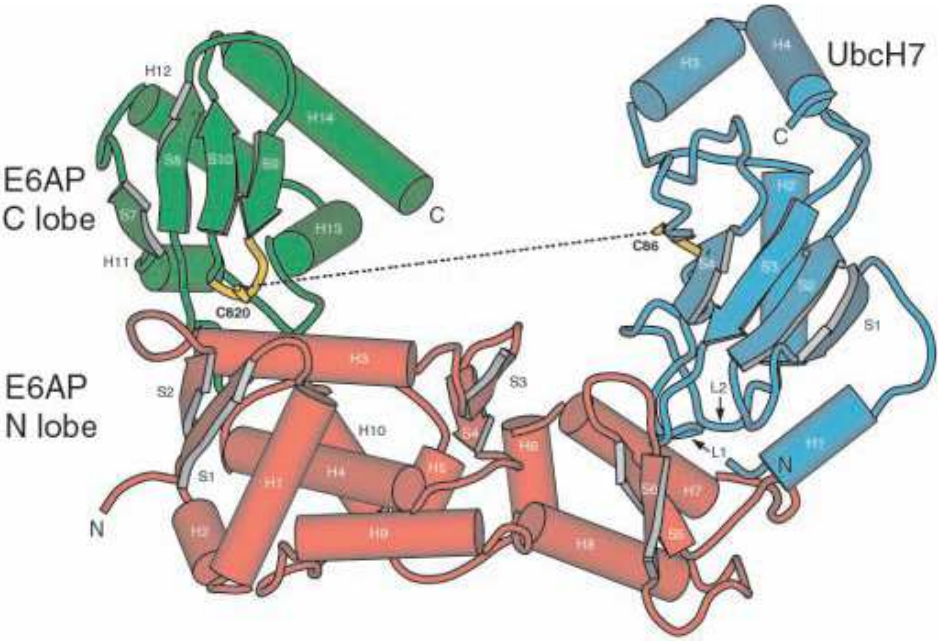


Figure 1.1 The E6AP HECT – UbcH7 complex forms a U-shaped structure. The E6AP HECT N-lobe, C-lobe and UbcH7 are in red, green and cyan, respectively. The active site loops are in yellow. The dotted line represents the distance between the two active site cysteines, which is 40 Å. Reprinted from Huang et al (68). Copyright (1999) with permission from AAAS.

The crystal structure of E6-AP HECT domain bound to UbcH7 revealed that the HECT domain has a bilobed structure with a short 3-residue hinge connecting the two lobes (Fig 1.1) (68). The N-lobe with its mostly α-helical structure has a V-shaped hydrophobic groove that pockets a highly conserved Phe residue on the E2. This interaction buries a total surface area of 1800 Å, with the Phe side chains making extensive van der Waals interactions with E6-AP residues. The C-lobe is smaller and bears the catalytic cysteine residue on an active site-loop, which makes contacts with both the N-lobe and C-lobe. A highly intriguing aspect of the crystal structure was the 41 Å distance between the active site cysteine residues of UbcH7 and E6-AP. Trans-thiolation reaction requires that the cysteine residues of the E2 and E3 are within 2.5 Å of each other (176). It was proposed that a major conformational shift might occur to bridge the gap between the E2 and the E3 (68). The crystal structure of the HECT domain of WWP1, a member of the Nedd4-like family of C2-WW-HECT proteins, showed a similar overall bilobed structure (176). However, the two structures differ in the orientation of the N-lobe with respect to the C-lobe. While the E6-AP HECT domain resembles an L-shape, with the C-lobe positioned at one end of the N-lobe, the WWP1 HECT domain resembles an inverted T-shape (⊥) (Fig. 1.2). This difference in orientation resulted in the active site cysteine of WWP1 being 16.5 Å apart from the active site cysteine of UbcH5, which was modeled into the structure. Further modeling studies on the flexible hinge between the N-lobe and C-lobe, reduced this distance to 5 Å. It was suggested that this flexible hinge allows the C-lobe, bearing the catalytic cysteine, to move towards the E2 cysteine residue during each cycle of transthiolation. The HECT domain of Smurf2, an

E3 that targets TGF-β receptors for degradation, was also crystallized (140). The Smurf2 HECT domain adopted an open conformation, similar to E6-AP, with an even greater distance between the active site cysteines (50 Å) of the E2 and E3 (Fig. 1.2). Despite these crystal structures, it is still not clear how a HECT domain transfers ubiquitin to substrates. Also, a full length HECT E3 has so far not been crystallized, and the orientation of the N-terminus with respect to the HECT domain is unknown. It is speculated that for each cycle of ubiquitin transfer, the C-lobe receives ubiquitin from an E2 at one side of the HECT domain and transfers it to the substrate bound to the N-terminus on the other side.

Most HECT domains have a conserved phenylalanine residue 4 amino acids before the stop codon (referred to as -4F). This residue was shown to be essential for transferring ubiquitin onto a ε-amino group of a substrate lysine by the HECT domain (161). Mutating the -4F did not affect thioester bond formation, but it abrogated ubiquitin transfer to the substrate. The position of the phenylalanine residue was also important, since it was shown that moving it to the -3 or -5 position did not restore function. It was proposed that the -4F might be important for correctly orienting the ubiquitin molecule while it is tethered by a thioester bond, thus allowing the substrate lysine to access the thioester bond.

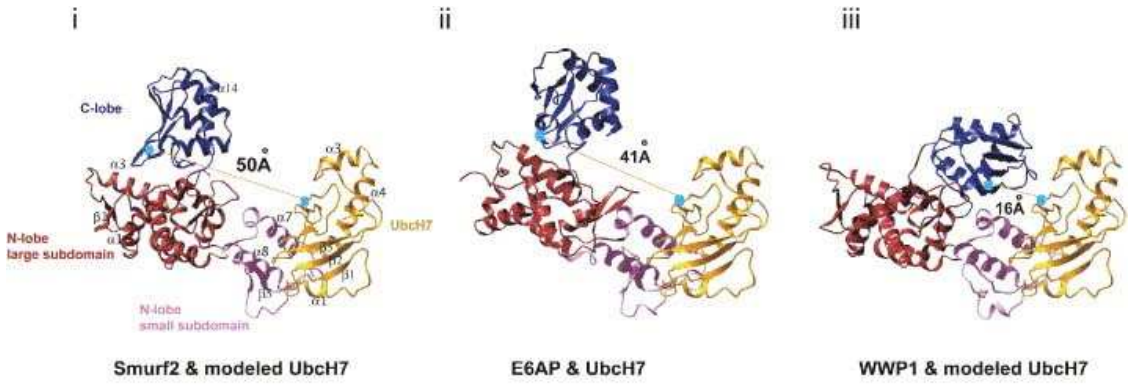


Figure 1.2 Comparison of the crystal structures of three HECT domains. As compared to the E6AP HECT domain, the Smurf2 HECT domain adopts a more open conformation, with the distance between the active site cysteines being 50 Å. The WWP1 HECT domain resembles an inverted T (⊥), as opposed to an L-shaped conformation of the other two HECTs. Reprinted from Ogunjimi et al (140). Copyright (2005), with permission from Elsevier.

Regulation of HECT E3s

Relatively few HECT E3s have been characterized with respect to their mode of regulation. Some HECT proteins, such as Herc5 and Herc6, are transcriptionally regulated (18) (101). Others are regulated by methods such as phosphorylation, regulation of E2 recruitment, or recruitment of deubiquitinating enzymes (90). Some of these modes of regulation are discussed below.

Although phosphorylation has been shown to change substrate affinities of HECT E3s (19), the study on ITCH was the first to show that phosphorylation can modulate the activity of a HECT ligase (38). ITCH is a C2 and WW domain-bearing HECT E3 that mediates the ubiquitination and degradation of the transcription factor JunB during T-helper2 (Th-2) cell differentiation. T-cell activation leads to JNK1-mediated phosphorylation of ITCH, which in turn, leads to activation of its catalytic activity (39). In the inactive state, the WW domains of ITCH bind to the HECT domain of this protein. This intramolecular inhibition is relieved when JNK1 phosphorylates ITCH at three sites within a proline rich region (PRR). Phosphorylation of ITCH causes a conformational change in the E3 that allows it to access its substrate JunB (38).

TGF-β signaling plays a role in many cellular processes involving growth and differentiation (156). This pathway is regulated at multiple steps, including ubiquitination and degradation of the TGF-β receptors by Smurf 1 and Smurf2, which terminates the signal. Smurf proteins, which belong to the C2-WW-HECT family of proteins, are recruited to the TGF-β receptors via their interaction with the adaptor protein SMAD7

(86). SMAD7 helps not only to localize the Smurfs but also to activate them. It was shown that the N-terminus of SMAD7 facilitates the interaction between the Smurf HECT domain and the cognate E2, UbcH7 (140). The crystal structure of Smurf2 revealed that it has a sub-optimal E2-binding pocket, with hydrophilic residues instead of the more typical hydrophobic residues seen in other HECT domains. SMAD7 overcomes this structural impediment by facilitating an interaction between the E2 and the HECT domain, thus increasing the activity of Smurf2.

The deubiquitinating enzymes (DUBs) USP8 and USP15 are associated with the RING E3s Nrdp1 and Rbx1, respectively (57, 185). They were shown to reverse the auto-ubiquitination of these ligases, thereby rescuing them from proteasomal degradation. The only HECT E3 that is known to interact with a DUB is Rsp5, an essential E3 in Saccharomyces cerevisiae. Rsp5 associates with Ubp2, a DUB, and the interaction is mediated by an adaptor protein Rup1 (91). Rsp5 preferentially adds K63 chains of ubiquitin onto substrates *in vivo* and *in vitro* (92). Ubp2 was shown to reverse K63-specific ubiquitin conjugation on Rsp5 substrates *in vitro*. Genetic evidence also indicates that Ubp2 antagonizes the function of Rsp5 *in vivo*. Physical association of a deubiquitinating enzyme with an E3 is a novel mechanism to regulate the activity of an E3.

HECT proteins and viruses

HECT E3s play important roles in several physiological processes and some of them are involved in fighting viral infections, whereas others are hijacked by viral proteins and aid viral replication. APOBEC3G is an antiviral molecule with cytidine

deaminase activity. It is packaged into retroviral particles as HIV-1 buds from the cells. The deaminase activity induces G-A mutations in the viral genome, many of which are lethal to the provirus (194). Nedd4-1, a HECT E3, ubiquitinates APOBEC3G and this serves as a signal to target the antiviral protein to the cell membrane, where it interacts with Gag and is subsequently packaged into the virus particles (24). The virus has evolved to counter this defense mechanism of the host. The Vif protein of HIV-1 interacts with cellular proteins to form a Vif-Cul5-SCF-like ligase that induces ubiquitination and degradation of the antiviral factor APOBEC3G (191). As part of my research, I discovered that a HECT E3 Herc5, is the major ligase for an antiviral ubiquitin-like protein, ISG15 and the results are described in Chapter 3 (18).

One of the best studied examples of viruses taking control of cellular machinery is that of the HPV protein E6, which alters the target specificity of the cellular HECT E3 E6-AP. High risk HPVs (e. g., HPV16, 18, 33 and 39) are associated with 95% of human cervical carcinomas, while the low risk HPVs are associated only with benign tumors (201). E6 can simultaneously associate with both E6-AP and p53, hijacking the ligase activity of E6AP and directing it towards p53 (71). As a result, p53 is ubiquitinated and targeted for proteasomal degradation.

The Epstein-Barr virus (EBV) latent membrane protein (LMP) 2A, plays a role in maintenance of viral latency by blocking B-cell signaling. The phosphorylation of LMP2A on its cytoplasmic domain recruits tyrosine kinases Syk and Lyn, via their SH2 domains (33). Further, the N-terminal region of LMP2A has PPPY motifs which interact with the WW domains of AIP4 and Nedd4-2. These ligases mediate the ubiquitination of

LMP2A-associated kinases Syk and Lyn, resulting in their down-regulation (183). Thus the viral protein acts as a scaffold to recruit HECT E3s and their substrates and disrupts B-cell receptor (BCR) signaling.

Many enveloped viruses exploit the activity of HECT E3s to stimulate ubiquitination of Gag proteins, an essential step for budding of viruses. A recent study reported that HECT domain proteins like WWP1 and WWP2 are recruited by viral proteins with PPXY motifs, to promote the budding of murine leukemia viruses (MLV) (118). Also, it was shown that Nedd4-1 catalytic activity was critical for the budding of human T-cell leukemia virus type 1 (HTLV-1). The HTLV-1 Gag protein contains a PPxY sequence which interacts with the WW domains of the HECT ligase Nedd4-1 and recruits it to the plasma membrane. Subsequently, Nedd4-1 ubiquitinates the Gag protein, a crucial event for viral release (10).

1.4 Ubiquitin-like proteins

A number of proteins have been identified that structurally resemble the three-dimensional β-grasp fold of ubiquitin. They are of two types: the ubiquitin domain proteins (UDPs) and the Ubiquitin-like proteins (Ubls) (178). UDPs have a ubiquitin-like domain in the context of a larger protein but they do not have a C-terminal glycine that can be conjugated to other proteins. The *S. cerevisiae* protein Rad23 is the best characterized example of this class. It has a ubiquitin-like domain at its N-terminus and two ubiquitin associated domains (UBA) in the latter part of the protein. The ubiquitin-like domain of Rad23 binds the Rpn1 subunit of the proteasome, whereas the UBA domains interact with polyubiquitinated proteins (14, 26). In this manner, Rad23 functions as an adaptor to recruit polyubiquitinated substrates to the proteasome. The other type of proteins that structurally resemble ubiquitin are the Ubls. Like ubiquitin, they are expressed as C-terminal extensions, which are processed to generate a C-terminal glycine residue. This glycine residue can form isopeptide linkages with lysine side chains on substrates (94). Each Ubl also has a distinct set of E1-E2-E3 enzymes, although in some cases, overlap with the ubiquitin enzymes has been observed (186, 196). Ubls and their enzymes have contributed to our understanding of the ubiquitin conjugation machinery. Crystal structures of Ubl enzymes have given us insights into the mechanisms of ubiquitin enzymes (177). About 10 Ubls have been discovered so far (55) and some of them and their enzymes are discussed below.

SUMO: Small Ubiquitin-related Modifier

SUMO shares ~18% identity with ubiquitin, yet its core structure is nearly identical to that of ubiquitin (8). There is a single essential SUMO gene in *Saccharomyces cerevisiae* (SMT3) but the human genome has three genes (SUMO 1-3). Like other Ubls, the first step in SUMO conjugation is catalyzed by an activating enzyme, which in this case is a heterodimer of Sae1 and Sae2 (81). Sae1 and Sae2 correspond in sequence to the N-terminus and C-terminus of Ube1, the E1 for ubiquitin. Ubc9 is the only known SUMO E2 enzyme (79). Ubc9 is distinct from other E2 enzymes in that it mediates substrate interaction. Most substrates are sumoylated on a conserved ΨKXD/E motif (where Ψ is a bulky aliphatic group and X is any amino acid), which is recognized by Ubc9 (9). Not all proteins that bear this motif are sumoylated, indicating that the structural context of this sequence is also important. Although *in vitro* E1 and Ubc9 are sufficient to transfer SUMO onto most substrates, efficient SUMO conjugation requires ligases or E3s (80). Also, in *S. cerevisiae*, almost all SUMO conjugation is dependent on E3s. SUMO E3s, like the RING E3s of the ubiquitin pathway, do not form covalent intermediates with the Ubl, but function by bringing the E2 in proximity to the substrate. Three classes of E3s have been recognized for the SUMO pathway. The first belongs to the Protein Inhibitor of STAT (PIAS) family of proteins, which bear an unusual RING-like motif. These proteins bind directly to Ubc9 and the substrates to stimulate SUMO conjugation. The second ligase is the vertebrate-specific protein RanBP2, which is a component of the nuclear pore complex (148). The 30 kDa catalytic core of RanBP2 contains neither RING domains nor HECT domains. It is not clear

whether RanBP2 is required *in vivo* for the sumoylation of proteins other than RanGAP1. The third type of SUMO ligase is Pc2, a member of the polycomb group of proteins (83). Polycomb proteins are involved in DNA methylation and epigenetic silencing of chromatin. CtBP, a transcriptional corepressor is recruited to the polycomb complex via Pc2 and Pc2 promotes sumoylation of CtBP both *in vivo* and *in vitro*.

Sumoylation plays a role in many diverse cellular pathways. At the molecular level, SUMO modification masks or adds a protein-protein interaction surface, thus altering function. SUMO modifications on transcription factors, in most cases, lead to transcription repression. In fact, translational fusions of SUMO to DNA binding proteins are sufficient to reduce promoter activity, suggesting that SUMO recruits repressors (78). Sumoylation is also involved in DNA repair via modification of the base excision repair enzyme, thymine DNA glycosylase (TDG). It was demonstrated *in vitro* that sumoylated TDG is capable of multiple rounds of catalysis as opposed to the unmodified enzyme. It is thought that SUMO modification reduces the affinity of the enzyme for its product, the AP site, and results in an increase in enzyme turnover (52).

In some cases, SUMO competes with ubiquitin to modify substrates, antagonizing the effect of ubiquitin conjugation. Lys 164 on proliferating cell nuclear antigen (PCNA) can be modified by ubiquitin as well as SUMO, resulting in different outcomes. Ubiquitination at this site is required to repair DNA lesions whereas SUMO modification at this site inhibits the damage-induced DNA repair pathway (51). In a similar manner, IκBα was reported to be sumoylated on the same lysine residue to which ubiquitin is conjugated. Ubiquitination of IκBα leads to its degradation, thus activating NFκB and the

inflammatory response pathway. Sumoylation, on the other hand, was proposed to block IκB degradation, and inhibit NFκB-mediated transcription (23).

RanGAP1 is the GTPase activating protein for the small GTPase Ran, which is involved in nuclear trafficking. RanGAP1 is the most abundant SUMO modified protein in vertebrate cells. The function of SUMO-RanGAP1 in nuclear transport is not yet clear, but *in vitro* nuclear import assays show that it is critical for the sumoylated form of the protein to be tightly associated at the nuclear pore complex (NPC). The SUMO E3, RanBP2 is localized on the cytoplasmic side of the NPC and the SUMO peptidase SENP2 is on its nuclear side. This has given rise to a model wherein proteins are rapidly sumoylated and desumoylated as they are shuttled into the nucleus (115, 120).

NEDD8/Rub1

NEDD8 shares the highest sequence identity with ubiquitin (~60%). Like SUMO, the E1 for NEDD8 is a heterodimer composed of two proteins, APPBP1 and UBA3 (45). The NEDD8 activating enzyme was the first E1 to be crystallized and its structure provided many insights into the mechanisms of ubiquitin/Ubl activation, E1-E2 interactions, and transfer of ubiquitin/Ubl from E1 to E2 (67, 177) . The crystal structure revealed that amino acid 72 of the Ubl is an important determinant for E1 specificity for this pair. Ubiquitin has an arginine at this position whereas NEDD8 has an alanine. A single amino acid substitution A72R, allows NEDD8 to form an efficient thioester bond with Ube1, while hampering thioester bond formation with its cognate E1 (177). Ubc12 was initially identified as the NEDD8 conjugating enzyme based on the observation that

deletion of Ubc12 and no other E2 abrogated NEDD8 conjugation in yeast. Roc1/Rbx1 and Mdm2 are suggested to be E3s for this pathway (128, 186). Both Roc1 and Mdm2 are RING domain proteins, but while Mdm2 is a single polypeptide with E3 activity, Roc1 is a component of the cullin-based E3s.

Until recently, the only known substrates for NEDD8 conjugation were the Cullins, which are the scaffolding proteins for the Cullin-RING ligases (CRLs). NEDD8 is ligated to a single conserved lysine residue on cullins, which activates the SCF ligase because it prevents the binding of CAND1 (cullin-associated and neddylation-dissociated-1), an inhibitor of SCFs (110). NEDD8 conjugation also boosts SCF activity by recruiting the ubiquitin conjugating enzyme to the cullin (89). It was also shown that neddylation of cullins makes them unstable and the activity of COP9 signalosome (CSN) is required to recycle them to the unneddylated, stable forms (184).

In 2004, Xirodimas *et al* showed that Mdm2, a RING finger ubiquitin ligase, promotes NEDD8 conjugation to the tumor suppressor p53 (186). It was deduced that neddylation inhibits the transcription activation function of p53, since p53 mutants that cannot be conjugated to NEDD8 display increased transcriptional activity compared to wild type p53. Mdm2 is itself auto-conjugated to NEDD8, but this does not affect its ability to ubiquitinate p53.

NUB1 (NEDD8 ultimate buster 1) was identified as a negative regulator of NEDD8 conjugation. NUB1 is a ubiquitin like protein that interacts with Rpn10/S5a subunit of the 19S proteasome subunit. NUB1 is thought to act as an adaptor to recruit neddylated proteins to the proteasome for degradation (171). It was also shown that

NEDD8 conjugated as the fourth subunit onto a K48 linked ubiquitin trimer, can directly bind to the 26S subunit of the proteasome (181). This result suggests that NEDD8 has all of the determinants necessary for targeting to the 26 S proteasome in the context of a Lys-48-linked chain.

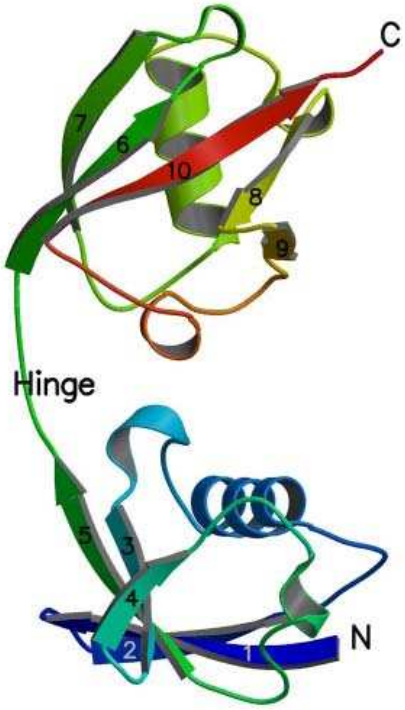
Not surprisingly, the NEDD8 pathway is essential for cell cycle progression, since many SCF targets are regulators of cell cycle. In fission yeast, disruption of NEDD8 conjugation is lethal, and Uba3-/- mice are not viable (173). The mutant mice embryos showed aberrant expression of p27Kip1, CyclinE and β-catenin, all substrates of the SCF ubiquitin ligases. Further, accumulation of NEDD8 has been observed in a number of neurodegenerative disorders such as Alzheimer's disease and Lewy bodies of Parkinson's disease (127).

Interferon-Stimulated Gene 15

ISG15 is one of the genes to be induced early in response to interferons α and β (31). It encodes a 17 kDa protein with two ubiquitin-like domains (48) and was shown to have anti-viral function (106). The biochemical functions of ISG15 modification are not known, although it was the first Ubl to be discovered. Some of the reasons for the slow progress in our understanding of this Ubl are that ISG15 is found only in vertebrates, making genetic approaches difficult. Also, until recently, the enzymes involved in ISG15 conjugation and the substrates that are modified by ISG15 were not known.

The crystal structure of ISG15 (Fig. 1.3), determined at 2.4 Å resolution, shows two ubiquitin-like domains with a six residue linker connecting them (135). Both domains of ISG15 maintain the β-grasp fold that is typical of the ubiquitin structure. The two ubiquitin-like domains bury 627 Å surface area between them, corresponding to 7% of the total solvent accessible surface of the protein. The contact surface between the two domains involves mainly van-der-Waals interactions and a weak hydrogen bond. Although this is thought to be the most stable solution structure, the authors concede that it is possible for the relative orientation of the two domains to change when ISG15 interacts with other proteins.

A.



B.

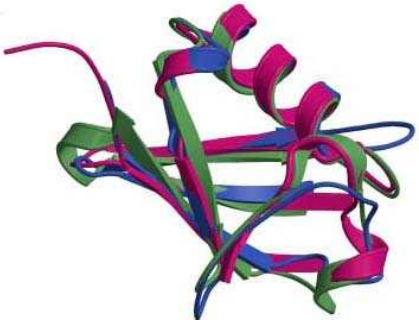


Figure 1.3 Structure of ISG15 resembles that of ubiquitin. A) A ribbon diagram of ISG15, showing the two ubiquitin-like domains connected by a flexible linker. B) An overlay of the ribbon diagrams of ubiquitin (pink) and the N- terminal (blue) and C-termainal (green) domains of ISG15. All three structures show the characteristic β-grasp fold. Reprinted from Narasimhan et al (135). Copyright (2005) with permission from ASBMB.

As with ubiquitin, a set of three types of enzymes, acting in sequential order, are responsible for the conjugation of ISG15 to its substrates. The Krug lab identified Ube1L, an enzyme highly homologous to the E1 of ubiquitin, as the E1 for ISG15 (193). Biochemical studies identified UbcH8 as the E2 for ISG15, a result that was confirmed by siRNA experiments (196). UbcH8 has been reported to be an E2 for ubiquitin as well (102, 130, 175, 195), suggesting possible cross-talk between the ubiquitin and ISG15 pathways. However, biochemical studies in our lab suggest that UbcH8 is not an efficient E2 for the ubiquitin system. The Km value of Ube1L for UbcH7 was estimated to be at least 50 times higher than for UbcH8, while the Km for UbcH8 was comparable to the values for other known E1-E2 pairs (Durfee L and J.M.H. - submitted to J Biol Chem). Both Ube1L and UbcH8 enzymes, like ISG15, are induced at the transcriptional level by-IFÑ-β. A major focus of my work has been to identify the ligase for ISG15. Herc5, a HECT E3, was determined to be a major ligase for this pathway and these results are described in Chapter 3.

Ubp43 (Usp18 in humans) was identified as the protease that cleaves ISG15 from ISG15 conjugated substrates. Although Ubp43 has several key residues that are conserved in ubiquitin-specific proteases (USPs), and it was shown to cleave Ub-β-gal fusions in Escherichia coli (111), it was later reported to be an ISG15 specific protease (117). Ubp43-/- mice are hypersensitive to type I interferons and exhibit neurological defects. Initially, the cause of these physiological defects was attributed to the accumulation of ISG15 conjugates in the brain. However, a double knockout mouse

lacking both Ubp43 and ISG15 genes still showed the same abnormalities and it was therefore concluded that the Ubp43-/- phenotype was not related to ISG15 (100). Also, since the Ubp43-/- mice showed ISG15 conjugates, it is clear that Ubp43 is not responsible for processing the inactive ISG15 precursor. When the C-termius of ISG15 was derivatized with a Vinyl-sulfone residue (ISG15-VS), it reacted with USP5, a previously known deubiquitinating enzyme, but not with Ubp43 (54). This method of tagging Ubls with VS is used to identify enzymes that react with a given Ubl. Given the above information, it is not clear whether Ubp43 is an actual ISG15 deconjugating enzyme, and if so, whether it is the only ISG15 deconjugating enzyme.

A large scale proteomics experiment identified the targets of ISG15 conjugation in IFN-β-treated HeLa cells using affinity purification followed by mass-spectrometry (197). The substrates identified included interferon-induced as well as constitutively expressed proteins, functioning in a range of cellular activities. There were a sizeable number of proteins involved in RNA processing and chromatin remodeling, suggesting that ISG15 might play a role in regulating transcription and/or RNA splicing events during microbial infections. It was not surprising that Ube1L, UbcH8 and Herc5 were among the substrates identified, since enzymes involved in the conjugation of Ub/Ubls, are often auto-conjugated (21, 145). Further, JAK1 and STAT1, the proteins involved in signal transduction from the interferon receptors, were also modified.

The consequences of ISG15 modification are not clear, but it appears that targets are not directed to the proteasome (116) and in fact, some substrates have been reported to be stabilized by ISG15 conjugation. An example is the transcription factor IRF3,

whose activity is tightly controlled by ubiquitin mediated degradation (114). In interferon-treated cells, IRF3 was shown to be conjugated to ISG15. This resulted in increased stability of the protein, which led to elevated levels of transcription activation. There have also been isolated reports of ISG15 modified proteins showing a decrease or increase in activity. The protein phosphatase 2Cbeta (PP2Cbeta), which is involved in NFκB pathway, was shown to have a decreased activity when it was conjugated to ISG15 (169). 4EHP, an mRNA cap-binding protein and a suppressor of translation, was reported to be modified by ISG15 via the RING-type ligase HHARI and this increased its cap-binding affinity (142) . However, the data suggested that the increase in cap-binding affinity was not significant.

As with SUMO, it has been observed that ISG15 is conjugated to only a small percentage of the total pool of a given subsrate. It is difficult to explain how ISG15 can modulate the function of a substrate if only a small fraction of the substrate is modified. SUMO is similarly conjugated to a low percentage of most substrates. A model has been proposed to explain SUMO conjugation (52, 78), which might also hold true for ISG15. In the case of thymine DNA glycosylase (TDG), SUMO conjugation is required to decrease its affinity for its product, an abasic site, thus allowing multiple rounds of catalysis. Once it is dissociated from the AP site, TDG is deconjugated to prepare it for the next round of reaction. In this way, even though very little TDG is modified at any given time, the whole population is affected by sumoylation. This model can be applied to other proteins. For example, a protein might need to be conjugated to become part of a protein complex, but the conjugation status of the protein may not be important once it is

within the complex. Thus it was proposed that a cycle of conjugation and deconjugation might be occurring such that conjugation is required to change the status of a protein, but not to maintain it. At any given time, even though two molecules of a substrate may be unconjugated, their functions might be different based on their conjugation histories.

ISG15 as an anti-viral molecule

It has long been suggested that ISG15 plays an antiviral role since it is one of the proteins that is turned on early during interferon responses. This was supported by the fact that the NS1 protein of influenza B virus binds ISG15 and blocks its conjugation to cellular proteins (193). The NS1B protein was shown to inhibit the ISG15~Ube1L thioester bond *in vitro*, preventing Ubl activation.

The most convincing demonstration of ISG15 antiviral activity came from experiments by H.W. Virgin's group. ISG15 expressed in mice lacking the interferon α/β receptor (IFNAR-/-) was shown to rescue Sindbis virus lethality (105). Further, the last six amino acids, LRLRGG, were shown to be important for mediating protection against the infection, stressing the importance of conjugation of ISG15 to targets. In another paper, the same group also demonstrated that ISG15-/- mice were more susceptible to infections of influenza A virus, influenza B virus, herpes simplex virus type 1 and Sindbis virus (106). The increased susceptibility of these mice to Sindbis virus infection was rescued by expressing wild-type ISG15, but not the non-conjugatable form. Another report showed that ISG15 could produce the same effects as interferon treatment, that is, inhibit the budding of virions in HIV-1 infected cells (141). Knock-down of ISG15 by

siRNA treatment rescued virus replication in interferon-treated cells. These experiments clearly established ISG15 as an antiviral molecule against both RNA and DNA viruses.

Viruses have evolved ways to block ISG15 conjugation, an example of which is the NS1B protein mentioned above. Recently it was shown that OTU domains, first discovered in the ovarian tumor protein, have deISGylating activity. OTU domain proteins are a class of cysteine proteases, some of which display de-ubiquitinating activity. A classic example is that of the protein A20 which plays a role in down regulation of NF-kappaB activity by deubiquitinating K63 linked chains on the substrate RIP. Giannakopoulos et al have shown that OTU domains from Nairoviruses and Arteriviruses have the ability to de-conjugate ubiquitin as well as ISG15. Co-transfection of OTU domains into 293T cells along with the ISG15 conjugation machinery, led to a decrease in ISG15 conjugates. Further, co-expression of OTU domains along with ISG15 abolished the ISG15-mediated viral resistance in IFNAR-/- mice. Thus, viruses have evolved such that their OTU domains can de-conjugate ISG15 as a means to evade the host immune response.

1.5 Innate Immune response

Innate immunity is the non-specific or generalized response of the body to the presence of pathogens such as virus or bacteria. It is the first line of defense against these micro-organisms and is responsible for their early detection and elimination, giving the body enough time to prime its adaptive immune system. It involves (i) the recognition of pathogen-associated molecular patterns by a set of molecules called pattern

recognition molecules (PRRs) (ii) the initiation of a downstream cascade of signals that result in the transcription of pro-inflammatory cytokines and type I interferons (164). The type I interferons, such as IFN-α and IFN-β, have anti-viral and anti proliferative functions and play a central role in mounting the innate immune response (62).

Toll-like receptors and downstream signaling

In order to have an effective response, the immune system needs to efficiently detect the invading micro-organisms. One major class of PRRs are the toll-like receptors (TLRs) that are encoded by cells of the innate immune system such as dendritic cells. First discovered in Drosophila where they mediate anti-fungal innate immunity, TLRs are the principal receptors that recognize molecular motifs associated with bacteria, fungi and viruses. There are 11 members in the TLR family, TLR1-11, recognizing diverse microbial signature molecules such as lipopolysaccharides (LPS), flagellin, dsRNA, ssRNA and unmethylated CpG DNA (1). They may be located on the cell surface where they mainly recognize bacterial components such as LPS, or within endosomes where they target viral DNA products.

All TLRs have an extracellular leucine-rich repeat domain (LRR), a single transmembrane domain and a Toll-Interleukin1 receptor (TIR) domain. The LRR mediates recognition of the pathogen-associated molecular patterns, with each TLR recognizing a specific microbial molecule (2). For example, TLR3 recognizes viral dsDNA while TLR5 is specific for flagellin. The TIR domain recruits downstream adaptors to transduce signals, which culminate in the activation of NF-κB and other

transcription factors. Signaling from all TLRs except TLR3 involves the Myeloma Differentiation factor (MyD88), a TIR domain containing adaptor protein that recruits downstream kinases IRAK1 and IRAK4 and the RING-type ubiquitin ligase TRAF6. TRAF6, along with the ubiquitin conjugating enzymes Ubc13-Uev1A, forms K63-linked ubiquitin chains which activate the kinase complex consisting of TAK1, TAB1 and TAB2 (20, 179). TAK1 activates IκB Kinase (IKK), resulting in the activation of NF-κB. The IL-1 receptor associated kinases (IRAKs) can activate IRF7, which leads to the induction of type I interferons. TLR3 is the only receptor that uses a MyD88-independent pathway that involves TLR-associated activator of interferon (TRIF) and the IKK-like kinases – TBK1 and IKKε, which activate transcription factors IRF3 and IRF7 (189).

All TLRs activate a set of signaling molecules that result in the activation of transcription factors NF-κB and AP-1 (a heterodimer of activating transcription factor 2 with c-JUN). These lead to expression of genes required for inflammatory and adaptive immune responses, such as IL-6, and tumor necrosis factor (TNF). Additionally, TLRs 3,4,7,8 and 9 also activate interferon regulatory factor-3 (IRF3) and/or IRF7 (87). The latent IRFs are cytosolic and upon phosphorylation, they dimerize and translocate to the nucleus. All these transcription factors (NF-κB, AP-1, IRF3 and/or IRF7) along with the high mobility group protein1 (HMG-1) assemble into a complex called the enhanceosome (97) which binds to the promoter of IFN-β gene and initiates transcription. IFN-β is secreted out of the cell and acts in a paracrine and autocrine manner to initiate a

cascade of events that lead to the expression of hundreds of ISGs, which in turn induce an anti-viral state in the host.

A type of pattern recognition receptors that are different from the TLRs are the retinoic acid inducible gene I (RIG-I) like helicases. These proteins have a DExD/H box class of helicase domain that recognizes viral dsRNA (190). The N-terminus has two caspase recruitment domains (CARDs) that mediate downstream signaling. Binding of viral dsRNA induces a conformational change that allows the RIG-I CARDs to form homotypic interactions with CARDs from a mitochondrial anti-viral signaling protein (MAVS, also called IPS-1/VISA/ CARDIF) (88, 124, 165, 188). MAVS activates the kinases responsible for activation of IRF3 and NF-κB, which in turn leads to production of IFN-β.

Type I interferons

Historically, interferons were molecules that were able to 'interfere' with viral replication and establish an anti-viral state. Type I interferons include IFN-α, IFN-β and seven other classes of IFNs (146). There is a single gene for IFN-β, whereas there are 20 genes that encode 13 subtypes of IFN-α. The IFN-α genes are clustered on one chromosome and are thought to be a product of gene duplication. The various sub-types have minor amino-acid differences, but differ in their expression profiles and the subset of genes they activate. Almost all cell types produce IFN α and β, but plasmocytoid

dendritic cells (pDCs) or 'natural IFN-producing cells' secrete the highest amounts of type I interferons (168).

There is a common receptor for IFNs α and β that is ubiquitously expressed on cell surfaces. It is composed of two subunits - IFNAR1 and IFNAR2 (138). IFN-β binding causes the two subunits to dimerize and begin a signal transduction cascade that culminates in the induction of several hundred Interferon Stimulated Genes (ISGs). Tyk2 and JAK1 are two members of the Janus tyrosine kinases that are associated with IFNAR1 and IFNAR2 respectively. Dimerization of the receptor leads to Tyk2 phosphorylation, which in turn phosphorylates JAK1. The two activated kinases phosphorylate the IFNAR1 subunit of the receptor, to create a docking site for Signal Transducer and Activator of Transcription-2 (STAT-2). STAT2 binds phosphorylated IFNAR1 via its SH2 domain, and recruits STAT1 (107). The two proteins form a heterodimeric complex that dissociates from the receptor and moves to the nucleus. Within the nucleus, the STAT1-STAT2 heterodimer associates with IRF9/p48 to form a transcription complex ISGF3 (interferon-stimulated gene factor 3). This multisubunit complex activates IFN-α and other genes with the interferon stimulated response element (ISRE) in their promoters (166). Knockout mice reveal the importance of the IFN α/β receptor in establishing the host response to viruses. These mice are extremely sensitive to infections by Poxviridae, Arenaviridae, Rhabdoviridae and Togaviridae family of viruses (74, 131, 160).

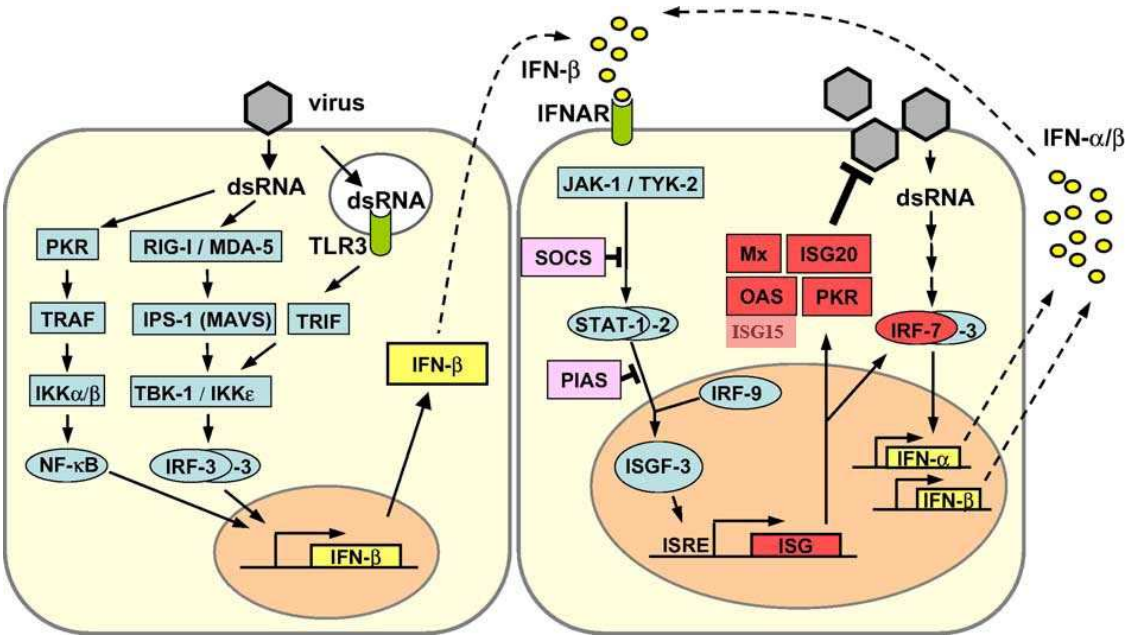


Figure 1.4 Type I IFN induction, signaling and action. Left panel: dsRNA, a characteristic by-product of virus replication, leads to activation of the transcription factors NF-κB, IRF-3 and AP-1 (not shown). The cooperative action of these factors is required for full activation of the IFN-β promoter. IRF-3 is phosphorylated by the kinases IKK(and TBK-1 which in turn are activated by the RNA-sensing complex of RIG-I, MDA5 and IPS-1/MAVS. A second signaling pathway involves endosomal TLR-3 and TRIF. Right panel: Newly synthesized IFN-h binds to the type I IFN receptor (IFNAR) and activates the expression of numerous ISGs via the JAK/STAT pathway. IRF-7 amplifies the IFN response by inducing the expression of several IFN-h subtypes. SOCS and PIAS are negative regulators of the JAKSTAT pathway. Mx, ISG20, OAS and PKR are examples of proteins with antiviral activity. Adapted from Haller *et al* (50). Copyright (2006), with permission from Elsevier.

Interferon regulatory factors (IRFs)

IRFs are the transcription factors responsible for modulating the expression of interferons and interferon stimulated genes (ISGs). There are nine IRFs encoded in the human genome, with IRF1 being the first family member to be discovered (61). Of these, IRF3 and IRF7 are involved in the early response to microbial infections. IRF9 complexes with STAT1-STAT2 to form the complex known as ISGF3 that initiates transcription of IFN stimulated genes. Some, such as IRF2 and IRF4, act as repressors of interferon responsive genes. Other IRFs regulate the extent of induction of IFNs and pro-inflammatory cytokines.

All IRFs have a conserved N-terminal DNA binding domain that forms a helix turn helix. Crystal structure of IRF2 bound to DNA revealed that 5'AANNGAAA-3' is the consensus sequence recognized by all IRFs (151). The C-terminal regulatory region of all IRFs (except IRF1 and IRF2) consists of an IRF association domain (IAD), through which these proteins interact with each other as well as other transcription factors like STAT proteins. The crystal structure of the IAD of IRF3 revealed that the H1 and H5 helices form a condensed inactive structure, which could be a possible auto-inhibition mechanism (151). Phosphorylation at multiple sites relieves this interaction, allowing the IRFs to bind DNA and other transcription factors. Besides phosphorylation, other post-translational modifications are also used to regulate the activities of some IRFs. Acetylation of IRFs1, 2 and 7 was shown to modulate their activities, with IRF2 being acetylated in a cell cycle dependent manner (144). Activation of IRF7 involves K63 ubiquitination via TNF-receptor associated factor 6 (TRAF6), a ubiquitin ligase (144).

Some viruses are known to down-regulate IRFs as a means to circumvent the interferon response. The RTA factor of Kaposi's Sarcoma-Associated Herpesvirus (KSHV) and the NSP1 of Rota virus were shown to induce poly-ubiquitination and degradation of IRF7 and IRF3 respectively (6, 192). On the other hand, certain viruses take advantage of the transcription activation function of IRFs. IRF1 was shown to bind to an ISRE-like element in the genome of human immuno-deficiency virus-1 (HIV-1), which led to stimulation of transcription from the viral LTR (7).

Interferon Stimulated Genes (ISGs)

As part of the innate immune response, ISGF3, a complex of STAT1-STAT2 and IRF9, binds consensus DNA elements upstream of IFN α/β inducible genes. There are more than 300 interferon stimulated genes (ISGs), which mediate the antiviral effects of interferons (168). Some of these and their mechanisms of interference with the viral machinery have been described below:

PKR: dsRNA dependent protein kinase

PKR is one of the better-studied interferon-induced proteins that plays an important role in fighting viral infections (123). The N-terminal domain of PKR binds dsRNA, whereas the C-terminus has a kinase domain. Although some PKR is constitutively present in a cell, it is in an inactive form. Binding of dsRNA causes dimerization, auto-phosphorylation and activation of the kinase domains (36). The activated PKR phosphorylates the α-subunit of translation initiation factor 2 (eIF2α,

which blocks the eIF2B-mediated GTP exchange reaction. This renders eIF2 inactive and inhibits translation of most viral and cellular mRNAs (40). Many viruses have devised ways to block PKR function. One example is that of the NS1 protein of influenza A virus, which binds PKR and inhibits its activity (125).

2'-5'OAS-RNaseL system

The 2'-5'OAS-RNaseL system is another well-characterized pathway that is turned on by interferons to counter viral infections (82). 2'-5' oligo-adenylate synthetases (2'-5'OAS) are a small family of proteins that are induced by interferons and activated by dsRNA, a common by-product of viral infection. Upon binding dsRNA, these enzymes convert ATP molecules into unique 2'-5' phospho-diester-linked adenylate oligomers (abbreviated as 2-5A). RNAseL, an endoribonuclease that is constitutively present in a latent monomeric form, is activated by binding to 2-5A. Activated RNase L cleaves single stranded viral and cellular RNA molecules, thus inhibiting viral replication.

ADAR1: RNA-specific Adenosine Deaminase

ADAR1 is an interferon inducible RNA editing enzyme that targets cellular and viral double stranded RNAs (41). ADAR1 catalyzes the deamination of an adenosine to an inosine. The resultant I-U mismatch could potentially destabilize the structure of RNA. Furhter, inosine is recognized as a guanosine by the transcription and translation machinery of the cell, causing an A-U to G-C mutation in the genome. This system is used by the host to increase the diversity of certain neuron receptors, however in the

context of the virus, this could have a deleterious effect on the function of the viral protein. A-I modifications, the result of ADAR1 activity, have been observed in the genomes of multiple viruses (13).

Mx GTPases

The Mx family of GTPases belongs to the superfamily of large dynamin-like GTPases (167). These interferon-inducible GTPases are very powerful anti-viral agents. Knockout mice that lacked the IFN α/β receptor but were transgenically expressing the human MxA protein, showed resistance to a variety of viruses, indicating that MxA alone could provide resistance to viral infections, without any of the other interferon- inducible proteins (53). The GTPase activity resides in the N-terminus of the protein and is required for anti-viral function. Via the C-terminus, this protein self-assembles into large oligomeric complexes. It is thought that in the presence of viral infection, the C-terminus forms aggregates with nucleocapsid structures of the virus, thus blocking access of the nucleocapsid proteins from the rest of the cellular machinery. Although humans encode two IFN-inducible Mx proteins, MxA and MxB, only the former was shown to have anti-viral function. The MxA protein has been shown to endow resistance to a variety of human viruses including bunyaviruses, togaviruses, orthomyxoviruses, paramyxoviruses, rhabdoviruses and picornaviruses (49).

P56 family

Human P56 (HuP56), along with HuP54, HuP58 and HuP60 are a family of structurally related proteins that are up-regulated by IFNα and β, double-stranded RNA and virus infections. These proteins have multiple tetratrichopeptide (TPR) repeats, but no other identifiable motif throughout their lengths. These 34 amino acid TPR repeats are found in a number of other proteins where they interact with multi-protein complexes (17). The HuP56 protein binds to the eIF3e subunit of the eukaryotic translation initiation factor 3, which is a complex of 11 subunits (47). This inhibits the function of eIF3 and down regulates translation, thus blocking viral replication. HuP56 was shown to block translation from the IRES element in hepatitis C virus (180).

Some of the ISGs described above, PKR, MxA, HuP54, HuP56, HuP58 and HuP60 were also identified in the proteomics study as proteins that were modified by ISG15 (197).

CHAPTER 2: MATERIALS AND METHODS

Cell culture, plasmids and antibodies

HeLa, 293 and 293T cells were grown in Dulbecco's modified Eagle's medium (Cellgro, Mediatech, Inc) supplemented with penicillin-streptomycin and 10% fetal bovine serum. High5 cells were grown in Grace's insect cell medium (Biowhittaker, Lenzo), supplemented with penicillin-streptomycin and 5% fetal bovine serum. Constructs for expression of Ube1L, UbcH8, and ISG15 were described previously. A plasmid containing the complete Herc5/CEB1 open reading frame with a Myc-tag, was provided by M. Ohtsubo (Hiroshima University). The Herc5 open reading frame and mutants were subcloned into a pcDNA3 vector containing an amino-terminal tandem affinity purification tag (pcNTAP) or an amino-terminal HA tag. A full-length clone for Herc6 was provided by R. Kroismayr (Medical University of Vienna) and was sub-cloned to bear an N-terminus TAP-tag as well as an HA tag. A plasmid coding for IQGAP1 was provided by Dr. Martin Poenie (The University of Texas at Austin). Full length IQGAP1 as well as truncation mutants were sub-cloned into pcNTAP vector. A baculo-virus expression construct for purifying His-tagged IQGAP1 was provided by George Bloom (University of Virginia).

Anti-p56 antibody was provided by Ganes Sen (Cleveland Clinic), anti-MxA antibody by Otto Haller (University of Freiburg), and ISG15 antibody by Ernest Borden (Cleveland Clinic). Monoclonal anti-IQGAP1 antibody was purchased from BD

Biosciences. An antibody to detect V5-tagged proteins was purchased from AbD Serotec. Anti-flag M2 antibody was obtained through Sigma and the HA.11 monoclonal antibody through Covance. PAP (peroxidase-anti-peroxidase) antibody (Rockland Immunochemicals for Research) was used to detect TAP-tagged proteins.

siRNA and DNA Transfections

siRNAs were purchased from Dharmacon or Invitrogen. Wherever mentioned, the 'smartpool' of siRNAs from Dharmacon was used to target genes. The smartpool is a mixture of four siRNAs designed to target four different sequences within a gene. The sequences of the siRNAs of a Smartpool were not disclosed. Either Oligofectamine (Invitrogen) or X-treme transfect (Roche) was used to transfect siRNAs at a final concentration of 20 nM or 100 nM, as indicated in each experiment. Cells were subjected to siRNA treatment for 56-72 hrs. The sequences of siRNAs used in this study are given in Table 1.

DNA transfections were done in 6-well or 12-well plates, using either Genejuice (Novagen) or Lipofectamine 2000 (Invitrogen). Cells were harvested 48 hrs post-transfection. For large scale transfections involving 10cm plates, the calcium chloride method (46, 182) was followed.

Reverse Transcriptase (RT)-PCR and Microarray Analyses

Total RNA for RT-PCR was isolated using the PARIS kit (Ambion, Inc.). SuperscriptII reverse transcriptase (Invitrogen) was used for cDNA synthesis, which was

used as a template in subsequent PCR reactions. Initially, a range of PCR cycles was performed using cDNA from IFN-β-treated or untreated cells to determine the linear range of amplification for each gene, and these parameters were used for PCR. Microarray analyses were performed as described previously (93). Elements chosen for analysis were screened for several data quality standards, including minimum intensity and pixel consistency.

Detection of ISG15 conjugates

siRNA transfections were carried out as described above. 12-24 hrs post-transfection, cells were treated with IFN-β (1,000 units/ml; Berlex) for an additional 48 hours. Cells were then harvested and lysed in 1% NP-40 buffer (100 mM NaCl, 100 mM Tris pH 7.5, 1% Nonidet P-40, 1 mM Dithiothreitol, 10 μg/ml Aprotinin, 1 μg/ml Leupeptin and 10 μg/ml PMSF). 30 μg of total cell extract was separated on an SDS-PAGE, transferred to a nitrocellulose membrane and probed with anti-ISG15 antibodies to view total cell conjugates.

Alternatively, 12-24 hours after the siRNA treatment, cells were transfected with plasmids expressing Ube1L (0.125 μg), UbcH8 (0.125 μg) and HA/Flag-tagged ISG15 (0.25 μg), with or without Herc5 expressing plasmids (0.25 μg). When other E3s were tested, they were transfected at the same concentrations (0.25 μg). 48 hours post-transfection, cells were harvested. 30 μg of total cell extract was separated on an SDS-

PAGE, transferred onto a nitrocellulose membrane and probed with anti-HA or anti-Flag antibodies to detect ISG15 and its conjugates.

To detect ISG15 conjugates of individual substrates, plasmids expressing tagged substrates (0.25 μg) were co-transfected into cells along with Ube1L, UbcH8 and ISG15, with or without Herc5. In the case of 3X-flag p56, extracts were run on an SDS-PAGE and probed with M2-flag antibodies to detect p56 and its conjugated form. For V5-moesin and V5-TrxR1, extracts were subjected to immuno-precipitation using anti-V5 antibody and the precipitated proteins were then separated on an SDS-PAGE followed by western blotting with anti-V5. To detect ISG15 conjugates of interferon-induced substrates, cells were harvested 48 hrs post-interferon treatment and extracts were immunoblotted with either p56 or MxA antibodies.

Auto-conjugation of Herc5 and Herc6 was observed by transfecting TAP-tagged versions of the E3s along with Ube1L, UbcH8 and ISG15 and subjecting the extracts to immuno-blotting with anti-TAP antibodies.

In the case of IQGAP1, 10cm plates of 293T cells were transfected with a TAP-tagged construct of IQGAP1 along with plasmids expressing Ube1L, UbcH8, and ISG15 with or without Myc-tagged Herc5. 48 hours post-transfection, cells were harvested in 400 μl 1% NP40 buffer and lysates were bound to IgG-sepharose beads (GE Healthcare) for two hours. Beads were washed in 0.1% NP40 buffer, resuspended in 1X SDS buffer and loaded on a denaturing PAGE. Separated proteins were transferred onto a nitrocellulose membrane, and probed with anti-TAP antibody to detect IQGAP1 and IQGAP1 conjugated to ISG15.

Purifying Ube1L , UbcH8 and IQGAP1 from Insect cells

Baculo-virus constructs expressing GST-tagged Ube1L or UbcH8 were used to infect High-5 insect cells. 48 hours after infection, cells were harvested, lysed in 1% NP-40 buffer, and extracts were bound to glutathione-sepharose beads (Novagen) for 3 hours at 4 ^{o}C. Beads were washed with 1X PBS, 1% Triton-X and resuspended in precision protease cleavage buffer (Tris pH 7.5 25 mM, NaCl 50 mM, 0.1% Triton-X, 1 mM Dithiothreitol, 1 mM EDTA). The beads were incubated overnight with 2 units of precision protease (GE Healthcare) and the purified enzymes were obtained in the supernatant. A baculo-virus expression construct for purifying His-tagged IQGAP1 was provided by George Bloom (119). Wild type IQGAP1 was purified on a Ni-NTA His Bind Resin (Novagen), as per manufacturer's protocol.

Preparation of ^{32}P-labelled ISG15

The pGEX6p plasmid (GE Healthcare) was modified to include a cAMP-dependent kinase phosphorylation site immediately downstream of the precision protease cleavage site and upstream of the multiple cloning site. The ISG15 open-reading frame was then cloned into this construct. The GST-ISG15 fusion protein was expressed in Escherichia coli DH10β and purified by standard methods on glutathione sepharose. The beads were resuspended in cAMP-dependent kinase reaction buffer (40 mM Tris pH 7.5, 20 mM magnesium acetate) and incubated with 158 units of cAMP-dependent kinase (Promega) and 20 μCi of ^{32}P-γ-ATP for one hour at room temperature. Excess gamma 32P-γ -ATP was washed off with 1XPBS and the beads were incubated overnight with 2

units of precision protease in its appropriate buffer. ^{32}P-labeled ISG15 was recovered in the supernatant.

In-vitro ISG15 conjugation assays

In vitro ISG15 conjugation assays were performed in the presence of 10 mM Tris pH 7.5, 50 mM NaCl, 7.5 mM ATP, 10 mM $MgCl_2$, and 0.1 mM DTT. Ube1L, UbcH8 and ^{32}P-labeled ISG15 were prepared as described above. Extracts of 293T cells transfected with Herc5 or its C-A mutant, were used as the source of E3. In some cases, extracts of IFN-β-treated HeLa cells were substituted as the E3 source. Purified E1, E2 and labeled ISG15 were incubated in the reaction buffer at room temperature for 30 min, with or without the E3. Purified E6AP was used as a positive control in these assays. Reactions were stopped by adding 1X SDS-PAGE loading buffer without DTT to detect thioester bonds, or with DTT to detect substrate conjugation. Products were run on an SDS-PAGE and visualized by autoradiography.

In vitro protein interaction assays

Plasmids expressing TAP-tagged Herc5 or various mutants of Herc5 were transfected in 10cm plates of 293T cells. 48 hrs post-transfection, cells were lysed in 1% NP-40 buffer and extracts were bound to IgG-sepharose beads for 2 hrs at 4 ^{0}C. Beads were washed in 0.1% NP-40 buffer and the bound TAP-tagged Herc5 proteins were assayed for binding to purified IQGAP1. Reactions were carried out in Tris pH 7.5 25 mM, NaCl 50 mM and 0.2% NP-40 at 4 ^{o}C for 2 hrs. Beads were subsequently washed in

0.1% NP-40 buffer and resuspended in 1X SDS-PAGE loading buffer. IQGAP1 was detected by immunoblotting.

In vivo IQGAP1-Herc5 binding assays

Wild type IQGAP1 and truncation mutants were expressed as TAP fusions, along with Myc-tagged Herc5, in 10 cm plates of 293T cells. 48 hrs post-transfection, cells were lysed in 1% NP-40 buffer, diluted in TRIS 25 mM, NaCl 50 mM buffer, and extracts were bound to IgG-sepharose beads for 2 hrs at 4 ^{0}C. Beads were washed in 0.1% NP-40 buffer and loaded on an SDS-PAGE. Bound Herc5 was detected by immunoblotting with anti-Myc antibody.

	siRNA sequence	Gene targeted
1	CAACTCCTGCTCTGAGATA GTTGAGGACGAGACTCTAT	E6AP
2	TAAGAGCACTGACATGTTT ATTCTCGTGACTGTACAAA	Herc5 (A)
3	GGAAGTAGCATAACTGTCA CCTTCATCGTATTGACAGT	Herc5 (B)
4	GACACAAACTTAATTCCTA CTGTGTTTGAATTAAGGAT	Herc5 (C)
5	GAGACTATGTTTCTAAGTA CTCTGATACAAAGATTCAT	Herc5/6
6	TCACCCAGATTTATACTTA AGTGGGTCTAAATATGAAT	Herc6 (A)
7	GAAGTCGCCTGGTTAAAGA CTTCAGCGGACCAATTTCT	Herc6 (B)
8	TGAAAGAGATCACCCAACA ACTTTCTCTAGTGGGTTGT	Herc6 (C)
9	AGGGGTAGAACCAAAAACT TCCCCATCTTGGTTTTTGA	hTfr (control)
10	TGCCATGGATGAGATTGGA ACGGTACCTACTCTAACCT	IQGAP1(A)
11	Smartpool	IQGAP1 (B)
12	Smartpool	TRIM22
13	Smartpool	TRIM25

Table 1: Sequences of siRNAs used for this research.

Plasmid Name	Description
pcNTAP Herc5 pcHA Herc5 pcFLAG Herc5	Wild type Herc5 cloned in pcDNA3 with different tags.
CS2 + MT CEB1	Wild type Herc5 cloned in pCS2 + MT (Myc-Tag). Provided by M. Ohtsubo (Hiroshima University).
pcNTAP Herc5 C-A	C994A mutant of Herc5 cloned in pcNTAP.
pcNTAP Herc5 ΔF	Last two amino acids of Herc5 (FG) were deleted.
pcNTAP Herc5 C5-6	Last amino acid of Herc5 (G) was replaced by the last 8 amino acids of Herc6 (VSPMLTQS).
pcNTAP Herc5 HECT	HECT domain of Herc5 (aa 686 – 1024) cloned in pcNTAP.
pcNTAP Herc5 RCC	Herc5 residues 1-380 cloned in pcNTAP.
pcNTAP Herc5 ΔRCC	Herc5 residues 381-1024 cloned in pcNTAP.
pcNTAP Herc5 Δ150	Herc5 residues 157-1024 cloned in pcNTAP.
pcNTAP Herc5 Δ100	Herc5 residues 101-1024 cloned in pcNTAP.
pcNTAP Herc5 ΔSBB	Herc5 deleted of residues 554-579, cloned in pcNTAP.
pcNTAP Herc6 pcHA Herc6	Wild type Herc6 cloned in pcDNA3 with different tags.
pcNTAP Herc6 C-A	C985A mutant of Herc6 cloned in pcNTAP.
pcNTAP Herc 5/6	Herc5 with Herc6 HECT domain cloned in pcNTAP.
pcNTAP Herc 6/5	Herc6 with Herc5 HECT domain cloned in pcNTAP.
pcNTAP Herc4 pcFLAG Herc4	Wild type Herc4 cloned in pcDNA3 with different tags.

pcNTAP TRIM25 (EfP)	Wild type TRIM25/Efp cloned in pcNTAP.
pcHA-NTAP E6AP	Double-tagged E6AP cloned in pcDNA3.
pcHA ISG15	HA-tagged ISG15 cloned in pcDNA3.
His-3X-FLAG-ISG15	Double-tagged ISG15 cloned in pcDNA3. Provided by Zhao C (Krug Lab).
His-3X-FLAG-p56	Double-tagged HuP56 cloned in pcDNA3. Provided by Zhao C (Krug Lab).
V5-Moesin	V5-tagged Moesin cloned in pcDNA3. Provided by Zhao C (Krug Lab).
V5-TrxR1	V5-tagged TrxR1 cloned in pcDNA3. Provided by Zhao C (Krug Lab).
pGEX2T-IQGAP1	Wild type IQGAP1 cloned in pGEX2T. Provided by Dr. Martin Poenie (Univ. of Texas at Austin).
pGEX6p IQGAP1	Wild type IQGAP1 cloned in pGEX6p.
pcNTAP IQGAP1	TAP-tagged IQGAP1 cloned into pcDNA3.
pcNTAP IQ 1001-1657	IQGAP1 residues 1001-1657 cloned into pcNTAP.
pcNTAP IQ 1-525	IQGAP1 residues 1-525 cloned into pcNTAP.
pcNTAP IQ 526-1657	IQGAP1 residues 526-1657 cloned into pcNTAP.

Table 2: List of plasmids used in this study.

CHAPTER 3: HERC5, AN INTERFERON-INDUCED HECT E3 ENZYME, IS REQUIRED FOR CONJUGATION OF ISG15 IN HUMAN CELLS

3.1 Introduction

Type 1 interferons play an essential role in innate immunity. One of the many genes strongly activated by IFN-α/β encodes ISG15, a 17-kDa ubiquitin-like protein (Ubl) (31, 48). Like ubiquitin (Ub), Ubls are linked to target proteins via isopeptide bonds between their terminal carboxyl group and lysine side chains of target proteins (75). The fact that ISG15 is expressed and conjugated in IFN-α/β stimulated cells and lipopolysaccharide-stimulated cells implies that ISG15 conjugation is likely to be a component of the innate immune response. This is supported by the finding that ISG15 knockout mice are more susceptible to infections of influenza virus, herpes simplex virus and Sindbis virus (106) and the NS1 protein of influenza B virus blocks ISG15 conjugation (193).

The biochemical effect of ISG15 on target proteins is unknown, however the recent identification of a large number of target proteins (197) provides opportunities for determining both the function of ISG15 and its role in the innate immune response. Also essential for functional studies is the identification of the complete set of enzymes

required for ISG15 conjugation. As with Ub conjugation, it is presumed that a cooperating set of E1, E2, and E3 enzymes, in addition to possible accessory factors, will be required for ISG15 conjugation. The ISG15 E1 and E2 enzymes have been identified. Ube1L is a single-subunit enzyme 62% similar to the Ub E1 enzyme (193), and UbcH8 is the major, if not exclusive, E2 enzyme for ISG15 (196). The genes encoding both Ube1L and UbcH8 are, like ISG15, transcriptionally activated by IFN-α/β (193, 196), suggesting that the entire conjugation system might be coordinately regulated.

A candidate E3 enzyme for ISG15 conjugation emerged from mass spectrometry-based identification of ISG15 target proteins (197). Proteomics analyses of SUMO- and Ub-conjugated proteins have shown that enzymatic components of Ub/Ubl conjugation pathways are often auto-conjugated (21, 145), and consistent with this, Ube1L and UbcH8 were identified as ISG15-modified proteins. In addition, a single HECT E3, Herc5, was identified as an ISG15-modified protein, suggesting that this enzyme might also be a component of the ISG15 conjugation pathway.

Further, Herc5 belongs to the HECT family of E3 enzymes (70), some of which can functionally interact with UbcH8 (102, 196). We demonstrated previously that Rsp5, an essential HECT E3 of Saccharomyces cerevisiae, can interact with UbcH7 and UbcH8 *in vitro*, and can also catalyze Ube1L- and UbcH8-dependent conjugation of ISG15 *in vitro* (196). Similarly, human E6AP, which also functions with UbcH7 and UbcH8, can catalyze conjugation of ISG15 to p53 in the presence of the human papillomavirus E6 protein *in vitro* (S. Beaudenon and J. M. H., unpublished results). While there is no evidence to suggest that either E6AP or human homologs of Rsp5 normally function in

the ISG15 pathway *in vivo*, these experiments predicted that bona fide ISG15 E3 enzymes might share conserved functional domains and mechanisms with Ub E3 enzymes.

The genes for Ube1L and UbcH8 were, like ISG15, upregulated by IFN-β and it was proposed that the entire ISG15 conjugating machinery might be coordinately regulated. Microarray gene expression experiments revealed that Herc5 and a closely related gene Herc6, were the only two HECT E3s induced by interferon and, together with the other evidence stated above, I hypothesized that Herc5 might be involved in ISG15 conjugation.

I show here that Herc5 is required for the conjugation of ISG15 to a broad spectrum of target proteins *in vivo*. siRNAs targeting Herc5 show a dramatic decrease in overall ISG15 conjugation in human cells, abrogating conjugation to the vast majority of ISG15 target proteins *in vivo*. Further, wild-type Herc5, but not its C994A mutant, enhances substrate conjugation when co-expressed with Ube1L, UbcH8 and ISG15 in non-IFN treated cells. I also demonstrate that wild type Herc5, but not the catalytically inactive C994A mutant, can auto-conjugate ISG15 *in vivo*, and extract of cells transfected with wild type Herc5 support ISG15 conjugation *in vitro*. Together, these results indicate that Herc5 is the major E3 enzyme for the ISG15 conjugation machinery.

3.2 Results

siRNAs against Herc5 lead to a decrease in ISG15 conjugates in IFN-β-treated cells

Herc5 is one of six human Herc proteins (HECT and RCC1), defined by a carboxyl-terminal HECT domain and by one or more RLDs (RCC1-like domains) (59). Herc5 was a candidate for being a component of the ISG15 conjugation pathway based on the fact that it was identified as an ISG15-modified protein (197), and it belongs to the HECT family of E3 enzymes, some of which can interact with UbcH8 (102, 196). To determine whether Herc5 plays a significant role in overall ISG15 conjugation, two synthetic double-stranded siRNAs (designated 5A and 5B) were designed to target Herc5 mRNA. The Herc6 protein is 49% identical to Herc5, and two siRNAs (6A and 6B) were therefore designed to target Herc6, as well as one siRNA that would simultaneously target both Herc5 and Herc6 (5/6). RT-PCR confirmed that transfection of the Herc5- and Herc6-specific siRNAs reduced Herc5 and Herc6 mRNA levels, respectively, in IFN-β-treated HeLa cells (Fig 3.1A).

Because a Herc5-specific antibody was not available, we took advantage of our epitope-tagged (HA and TAP) Herc5 expression vectors to confirm that all three Herc5 siRNAs effectively knocked-down Herc5 at the protein level (Fig 3.1B). The 5A, 5B, and 5/6 siRNAs resulted in a dramatic reduction of HA-Herc5 protein, while neither of the Herc6 siRNAs, an siRNA against E6AP nor a UbcH8-specific siRNA, affected HA-Herc5 protein levels. Only the E6AP-specific siRNA affected the level of the endogenous

E6AP HECT E3. Fig 3.1C shows that siRNAs against Herc6 effectively lowered the levels of TAP-tagged Herc6, whereas the control siRNAs did not.

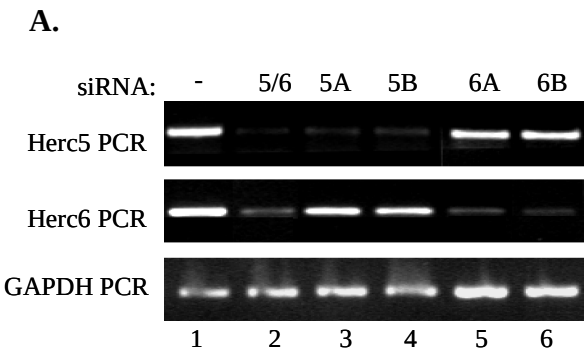


Figure 3.1 Herc5 and Herc6 siRNAs are effective at the mRNA as well as protein levels. A) RT-PCR analysis to determine the effects of siRNAs on Herc5, Herc6, and GAPDH (glyceraldeyde-3-phosphate dehydrogenase) mRNA levels in IFN-β-treated HeLa cells. Cells were transfected with siRNA targeting either Herc5 (5A, 5B), Herc6 (6A, 6B), or both Herc5 and Herc6 (6/5). Total RNA was isolated, and RT-PCR was performed with primers specific for Herc5, Herc6, or GAPDH.

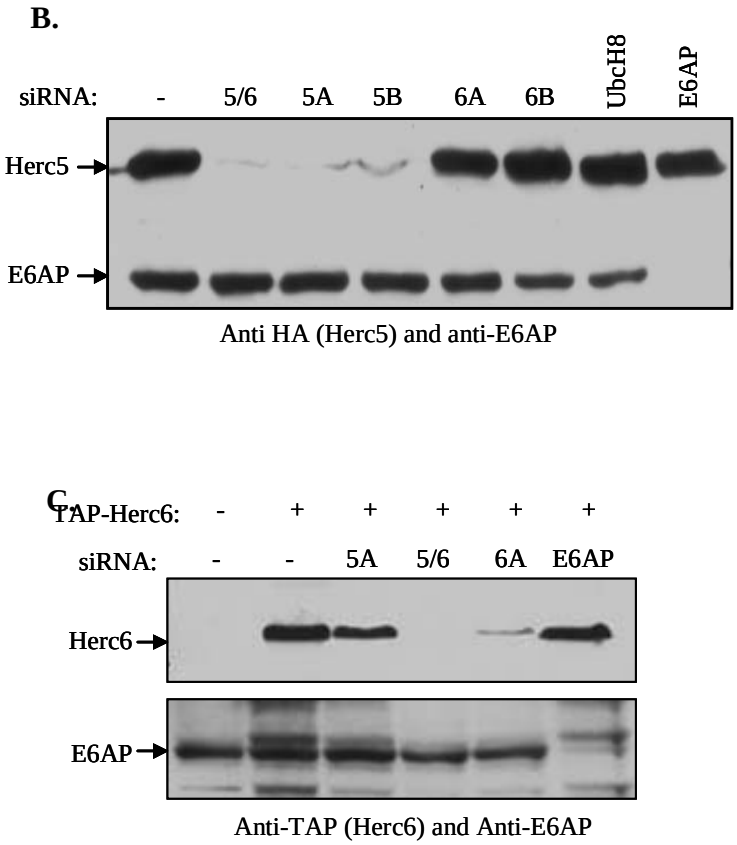


Figure 3.1 HeLa cells were transfected with the indicated siRNAs. Eight hours later, they were transfected with a plasmid expressing HA-tagged Herc5 (B) or TAP-tagged Herc6 (C). Cell extracts were prepared after an additional 48 h, and an immunoblot was performed with anti-HA antibody (B) or anti-TAP antibody (C) to detect levels of Herc5 and Herc6, respectively. E6AP was used as a control in both cases.

HeLa cells were transfected with the Herc5 or Herc6 siRNAs or, as a negative control, an siRNA against the E6AP HECT E3 (93). Six hours after transfection, IFN-β was added for an additional 48 hrs. Total cell extracts were prepared and ISG15 conjugates were analyzed by immunoblotting with an antibody against ISG15. Induction of ISG15 and high molecular weight ISG15 conjugates was observed in IFN-β-treated cells (Fig 3.2, compare lanes 1 and 2). Both of the Herc5-specific siRNAs and the siRNA that targeted Herc5 and Herc6 (5/6) resulted in a dramatic decrease in overall ISG15 conjugates (lanes 3–5), while neither of the Herc6-specific siRNAs or the E6AP siRNA led to a discernible decrease in ISG15 conjugates (lanes 6–8).

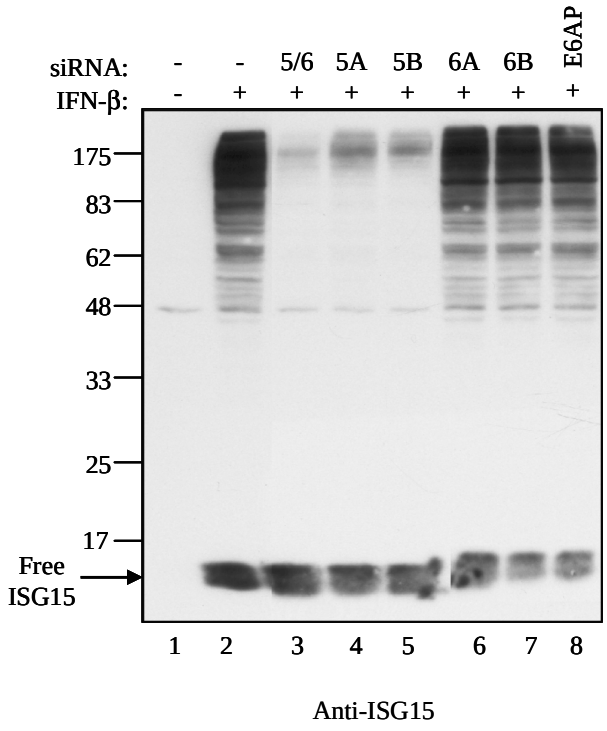


Figure 3.2 siRNAs against Herc5 lead to a decrease in ISG15 conjugates. HeLa cells were mock-transfected (lanes 1 and 2) or transfected with the indicated siRNAs and then treated with IFN-β for 48 h or left untreated (lane 1). l Article">17</ref-type><contributors><authors><auth or>Dastur, A.</autho15 antibody. Molecular weight markers are indicated (left).

Combinations of Herc5 and Herc6 siRNAs were also transfected together, on the premise that potential Herc6-dependent effects might be more evident following reduction of Herc5 activity (Fig 3.3). For these experiments, a third Herc5 siRNA (5C) was used that elicited only a partial reduction in Herc5 activity (compare lanes 1–3). Co-transfection of Herc6 siRNA 6A with either Herc5 siRNA 5A or 5C did not elicit any further decrease in ISG15 conjugates relative to the Herc5 siRNAs alone.

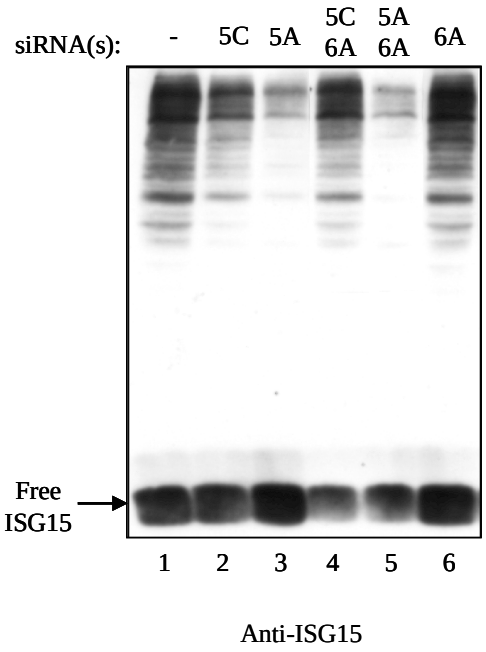


Figure 3.3 Co-transfection of Herc5-Herc6 siRNAs did not lead to a further decrease in ISG15 conjugates. HeLa cells were mock-transfected (lane 1) or transfected with individual Herc5 or Herc6 siRNAs (lanes 2, 3 and 6) or combinations of Herc5 and Herc6 siRNAs (lanes 4 and 5), and then treated with IFN-β for 48 hrs. Cell extracts were prepared and analyzed by immunoblotting with ISG15 antibody.

The effect of Herc5 siRNAs on conjugation of ISG15 to two specific target proteins, p56 and MxA, was also examined (Fig 3.4). Both these proteins are induced by IFN-β, and are anti-viral proteins (162). p56 binds to eIF3 and inhibits translation of viral proteins (47) and MxA is a large GTPase that inhibits viral replication (49). The identification and validation of the IFN-β -induced ISG15-conjugated forms of both of these proteins was described previously (197). Consistent with the effect of Herc5 siRNAs on total ISG15 conjugates, siRNAs that targeted Herc5 blocked conjugation to both p56 and MxA, while Herc6-specific siRNAs did not. We conclude that Herc5 plays a major role in mediating overall ISG15 conjugation to a broad spectrum of target proteins. Given that it is interferon-induced, it is possible that Herc6 might also function in the ISG15 conjugation pathway, but if so, it clearly plays a minor role relative to Herc5.

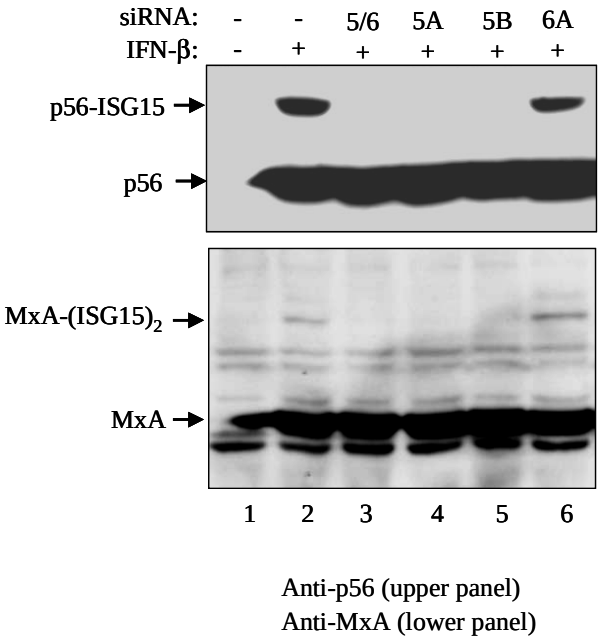


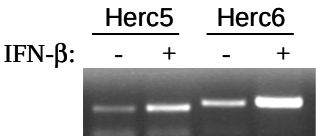
Figure 3.4 siRNAs against Herc5 block ISG15 conjugation of p56 and MxA. HeLa cells were mock-transfected (lane1) or transfected with siRNAs as shown and then treated with IFN-β for 48 hrs. Cell extracts were immunoblotted with antibodies against p56 and MxA.

Herc5 is induced by interferon-beta

The genes encoding ISG15, Ube1L, and UbcH8 are transcriptionaly induced in response to interferon signaling. We therefore determined whether expression of Herc5 and/or Herc6 was regulated by IFN-β by microarray gene expression analyses, comparing IFN-β-treated cells to untreated cells at various time points following addition of IFN-β (3, 6, and 21 h). Fig 3.5A shows representations of the microarray elements corresponding to ISG15, Ube1L, UbcH8, Herc5, and Herc6 cDNAs, along with elements corresponding to three genes not expected to be affected by IFN-β treatment (E1Ub, UbcH7, and E6AP). The induction of ISG15, Ube1L, and UbcH8 was evident, along with both Herc5 and Herc6, with maximal induction over this time course at 21 h post-IFN-β treatment. This corresponds to the beginning of maximal accumulation of ISG15 conjugates (112). Expression of E1Ub, UbcH7, and E6AP was not affected by IFN-β treatment. The results from micro-array analysis were confirmed by RT-PCR. Fig 3.5B shows that Herc5 and Herc6 are induced by IFN-β at the mRNA level.

The time course and magnitude of Herc5 and Herc6 induction was similar to that of Ube1L and UbcH8 (Fig 3.5C). The regulation of Herc5 and Herc6 expression by IFN-β is consistent with the demonstrated importance of Herc5 in ISG15 conjugation, as well as a potential minor role for Herc6.

A.



B.

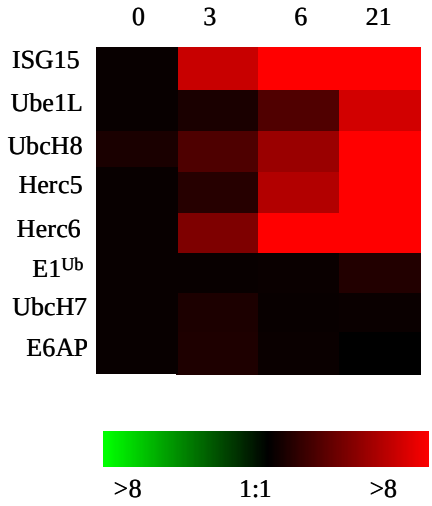


Figure 3.5 Herc5 and Herc6 expression is induced by interferon-β. (Figure legend on next page)

C.

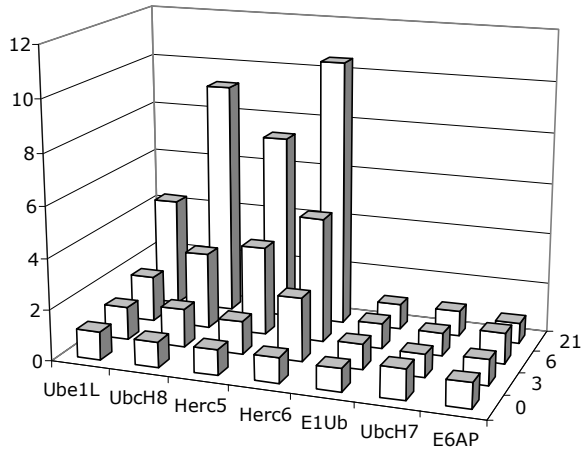


Figure 3.5 Herc5 and Herc6 expression is induced by interferon-β. A) RT-PCR analysis. Total RNA was prepared from non-interferon-treated and interferon-treated HeLa cells. RT-PCR was performed using Herc5 and Herc6–specific primers. B) Microarray gene expression analysis was performed, comparing HeLa cells treated with IFN-β for the indicated time periods (0, 3, 6, and 21 h) to untreated HeLa cells. cDNA from IFN-β-treated and untreated cells were labeled with Cy5 (*red*) and Cy3 (*green*), respectively. Microarray elements corresponding to the indicated genes are shown. C) Bar graph representation of the microarray data shown in B, illustrating the time course and magnitude of induction for each of the indicated genes.

Herc5 boosts ISG15 conjugation in non-IFN-treated cells

Co-transfection of plasmids expressing ISG15, Ube1L, and UbcH8 leads to ISG15 conjugation in non-IFN-treated HeLa cells, although at a lower level than observed in IFN-β-treated cells. To determine whether this level of conjugation was due to a significant basal level of Herc5 expression, I transfected Herc5 siRNAs prior to co-transfection of plasmids expressing ISG15, Ube1L, and UbcH8. As shown in Fig 3.6, transfection of Herc5 siRNAs (5A, 5B, or 5/6) did indeed block ISG15 conjugation, while Herc6 siRNAs did not, indicating that a basal level of Herc5 expression is responsible for ISG15 conjugation in this context. This is consistent with my finding that RT-PCR of the Herc5 gene from non-interferon induced cells shows a low level of Herc5 mRNA present in these cells (Fig 3.5A).

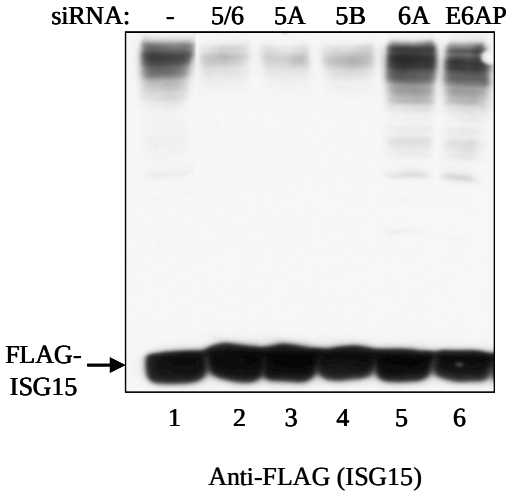


Figure 3.6 Basal level of Herc5 is responsible for ISG15 conjugation in non-interferon-treated cells transfected with E1, E2 and ISG15. Cells were first transfected with the indicated siRNAs and then transfected with plasmids expressing FLAG-ISG15, Ube1L, and UbcH8. Cell extracts were prepared and an immunoblot performed with anti-FLAG antibody.

I also determined whether co-transfection of a Herc5-expressing plasmid would enhance the level of ISG15 conjugates seen in non-IFN-treated cells. In both HeLa cells (Fig 3.7) and 293T cells (data not shown), transfection of a Herc5-expressing plasmid boosted ISG15 conjugation ~5-fold over that seen with transfection of ISG15, Ube1L, and UbcH8 plasmids. I constructed a Herc5 mutant in which the catalytic cysteine residue in the HECT domain was replaced by an alanine (C-A). This conserved cysteine residue is a hallmark of HECT type E3s and has been shown to form a thioester bond with ubiquitin prior to transferring it to the substrate. Importantly, the Herc5 active-site mutant (C994A) did not stimulate ISG15 conjugation, indicating that the catalytic activity of Herc5 is required for its function in the ISG15 system. Herc6 was also co-transfected along with the E1, E2 and ISG15 expressing plasmids. There was no change in the levels of ISG15 conjugates in the presence or absence of Herc6, again indicating that Herc6 does not affect overall ISG15 conjugation.

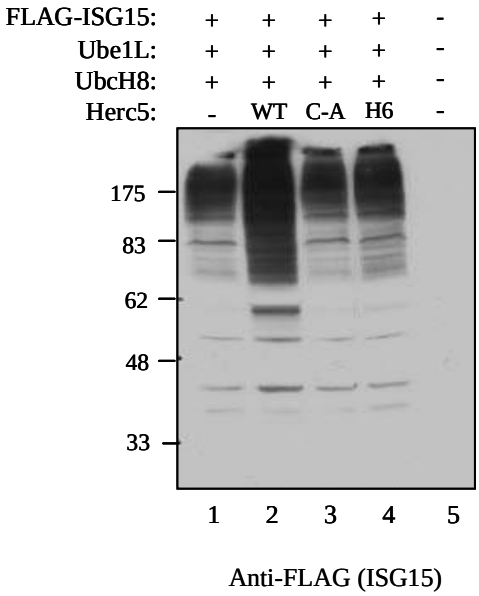


Figure 3.7 Herc5 boosts ISG15 conjugation in non-interferon-treated cells. HeLa cells were either mock-transfected (lane 5) or transfected with plasmids expressing FLAG-ISG15, Ube1L, and UbcH8, without Herc5 (lane 1) or with co-transfection of plasmid expressing wild type Herc5 (lane 2) or the active-site C994A mutant (lane 3) or wild type Herc6 (lane 4).

I examined the levels of ISG15 conjugates of two individual targets, p56 and Moesin, in the presence and absence of transfected Herc5. As mentioned above, p56 is a protein with anti-viral function. Moesin connects plasma-membrane proteins to actin filaments and is involved in mediating cytoskeletal changes (126), and it was recently shown to have anti-retroviral activity (133). HeLa cells were transfected with Ube1L, UbcH8, ISG15 and Herc5, along with FLAG-tagged p56 or V5-tagged moesin. Conjugation of both substrates to ISG15 was enhanced when wild type Herc5, but not the C994A mutant, was included (Fig 3.8, compare lanes 3 vs 4 and lanes 7 vs 8), confirming the results seen with overall ISG15 conjugation. Also, Herc6 did not boost ISG15 conjugation to either of these substrates.

UbcH8 is the only E2 enzyme known to function in ISG15 conjugation (196), and as expected, Herc5-dependent stimulation of ISG15 conjugation was not evident upon co-transfection with UbcH7 (Fig 3.9, lanes 2 and 5). In addition, a single amino acid mutation at residue 62 of UbcH8 (F62A) abrogated Herc5-dependent ISG15 conjugation. F62 of UbcH8 represents a conserved residue among E2 proteins that have been shown to interact with HECT E3s (e.g. UbcH5 isoforms, UbcH6, UbcH7, UbcH8), and both biochemical and structural experiments have indicated that this residue is critical for the ability of E2s to interact with the HECT domain (68, 139). The fact that the F62A mutation of UbcH8 abrogated Herc5-dependent ISG15 conjugation is consistent with a model in which UbcH8 directly interacts with Herc5 in mediating ISG15 conjugation.

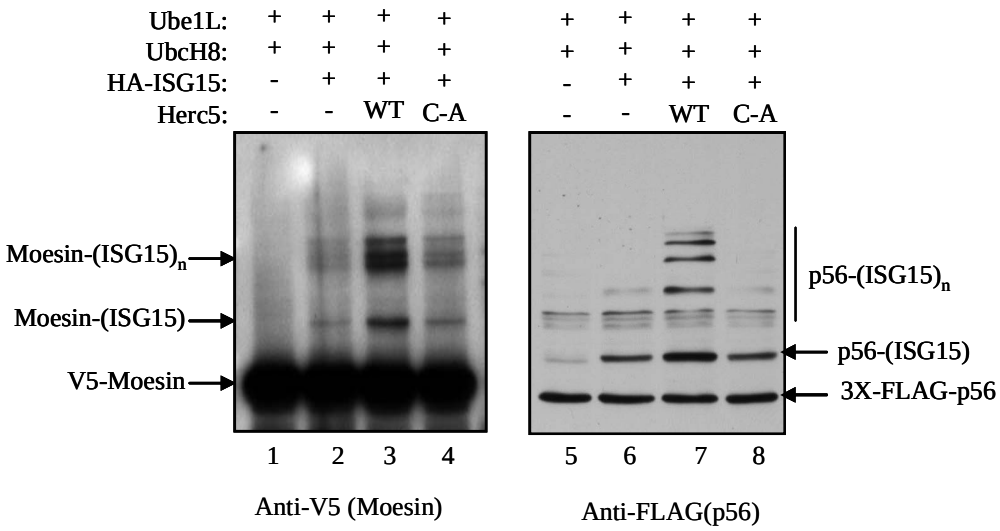


Figure 3.8 Herc5 boosts ISG15 conjugation of Moesin and p56 in non-interferon-treated cells. HeLa cells were transfected with V5-Moesin (left panel) or 3X-FLAG p56 (right panel), along with plasmids expressing Ube1L, UbcH8, ISG15 and no Herc5 (lanes2 and 6) or wild type Herc5 (lanes 3 and 7) or the C994A mutant (lanes 4 and 8). Cell extracts were prepared and immunoblots were probed with anti-V5 antibody (left panel) or anti-FLAG anti-body (right panel).

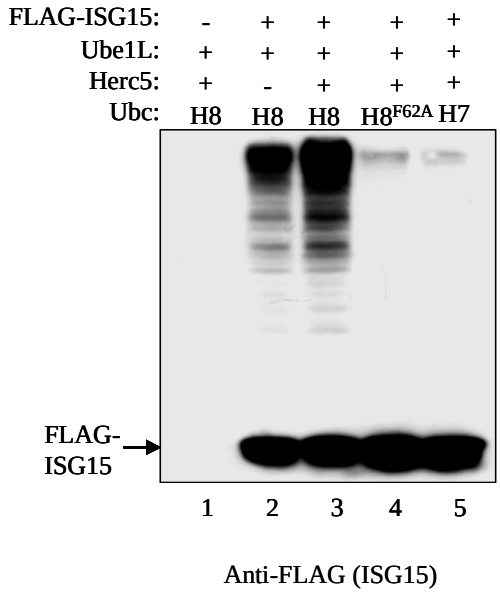


Figure 3.9 UbcH8, but not UbcH7, functions with Herc5 in vivo. 293 cells were transfected with plasmids expressing FLAG-ISG15, Ube1L, and UbcH8, without (lane 2) or with co-transfection of plasmid expressing wild-type Herc5 (lanes 3). Plasmids expressing the F62A UbcH8 mutant or UbcH7 were used in place of UbcH8 plasmid in lanes 4 and 5, respectively. Cell extracts were immunobloted with anti-FLAG antibody.

TRIM25 (EfP) does not affect overall ISG15 conjugation

Micro-array analysis showed that, in addition to Herc5 and Herc6, six other ubiquitin ligases were transcriptionally up-regulated by IFN-β. They all belong to the Tripartite Motif (TRIM) family of proteins, which are characterized by the presence of a RING domain, one or more B-box motifs and a coiled coil domain. Some of the TRIM members have RING-domain-dependent ubiquitin ligase activity (122), and at least one of these, TRIM25 (or Efp), has been shown to interact with UbcH8 (175). I determined whether TRIM25 might be involved in ISG15 conjugation, using similar approaches as described above for Herc5 and Herc6. A set of four siRNAs were designed to target TRIM25 (Smartpool from Dharmacon), and cells were transfected with a common mix of all four at a final concentration 100 mM. Figure 3.10 shows that the siRNAs efficiently knocked down TRIM25 at the protein level. Cells were transfected with the TRIM 25 siRNAs, followed by IFN-β-treatment for 48 hours. As seen in figure 3.11, knockdown of TRIM25 did not affect overall ISG15 conjugation. Further, TRIM25 did not enhance ISG15 conjugation when co-transfected with Ube1L, UbcH8 and ISG15 (Fig 3.12). Neither of these results suggest that TRIM25 plays a role in ISG15 conjugation.

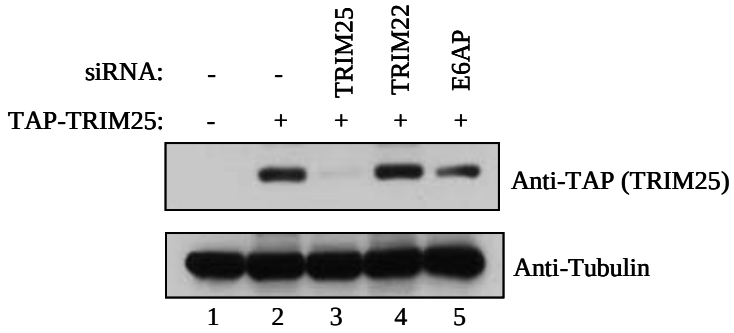


Figure 3.10 siRNAs against TRIM25 are effective at the protein level. HeLa cells were either mock transfected (lane 2) or transfected with siRNAs as indicated, and then transfected with TAP-tagged TRIM25. Cell extracts were immunoblotted with anti-TAP antibody to detect TRIM25 or anti-tubulin antibody as a control.

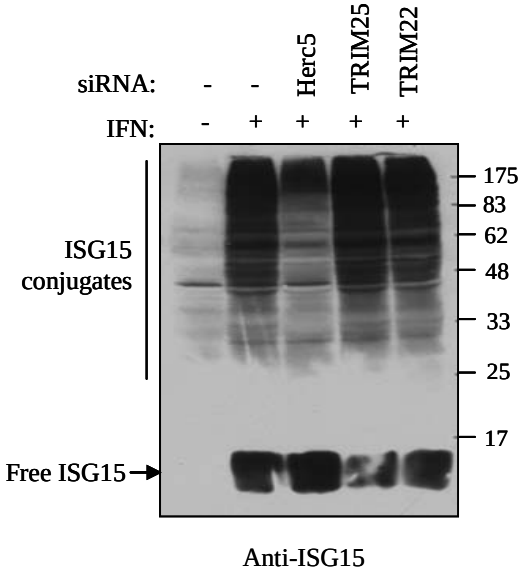


Figure 3.11 siRNAs against TRIM25 do not affect overall ISG15 conjugation. HeLa cells were either mock transfected (lane 2) or transfected with siRNAs against Herc5 or TRIM25 or TRIM22 and then treated with IFN-β for 48 hrs. Cell extracts were immunoblotted with anti-ISG15 antibody.

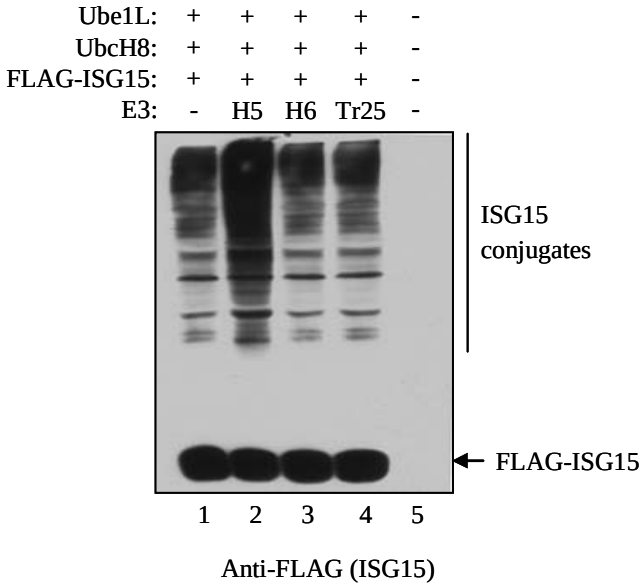


Figure 3.12 TRIM25 does not boost ISG15 conjugates in non-interferon-treated cells. HeLa cells were transfected with plasmids expressing Ube1L, UbcH8, FLAG-ISG15 with no Herc5 (lane 1) or with plasmids expressing Herc5 (lane 2) or Herc6 (lane 3) or TRIM25 (lane 4)

Herc5 is auto-conjugated to ISG15

A common feature exhibited by many ubiquitin E3 enzymes is auto-ubiquitination. The physiological significance of this phenomenon is not always known. For certain ligases, auto-ubiquitination leads to degradation and hence down-regulation of the E3 activity, as is the case with Mdm2 (29, 63). In other cases, auto-conjugation is required for activating downstream events, as seen with K-63 linked auto-ubiquitination of TRAF6 (104). Herc5 was one of the proteins identified in the ISG15 proteomics assay and I determined whether this was a result of auto-conjugation. Extracts of cells transfected with plasmids expressing Ube1L, UbcH8, ISG15 and TAP-Herc5 were probed with anti-TAP antibodies. High molecular weight ISG15-dependent conjugates of Herc5 were detected (Fig 3.13). No conjugates were observed when ISG15 was not included in the transfection mix (Fig 3.13, lane 2), ruling out the possibility that Herc5 might be auto-conjugated to ubiquitin. The majority of conjugates were detected only when UbcH8 was co-transfected (Fig 3.13, lanes 3 and 4). The small number of conjugates seen in the absence of transfected UbcH8 is likely due to the low levels of endogenous UbcH8. Further, the catalytically inactive C994A mutant of Herc5 was not modified, confirming that the conjugates were a result of auto-conjugation (Fig 3.13, lanes 5 and 6).

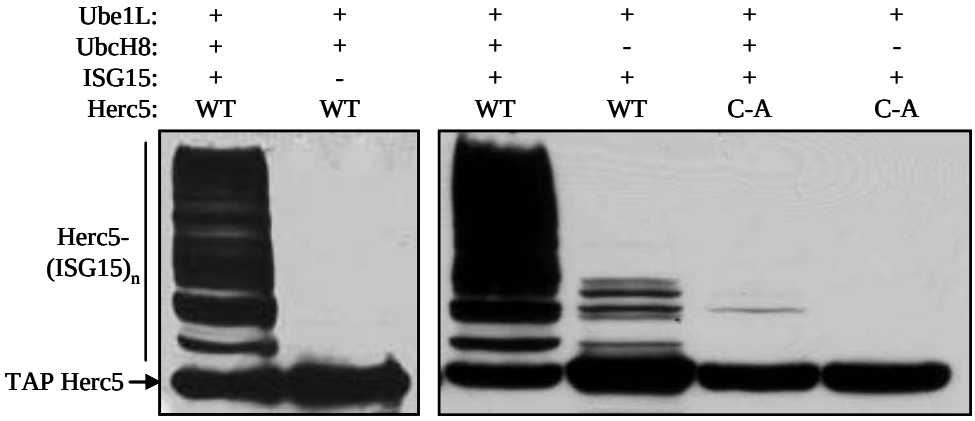


Figure 3.13 Herc5 is auto-conjugated to ISG15 in vivo. HeLa cells were transfected with wild type TAP-tagged Herc5, with or without ISG15 (lanes 1 and 2), and with or without UbcH8 (lanes 3 and 4). The C994A mutant was also transfected with or without UbcH8 (lanes 5 and 6). Cell extracts were prepared and immunoblotted with anti-TAP antibody to detect Herc5.

In-vitro assays for ISG15 conjugation

In order to demonstrate ISG15 conjugation activity of Herc5 *in vitro*, I purified each of the components of the ISG15 machinery. Ube1L and UbcH8 were expressed as GST-fusion proteins in insect cells and purified on glutathione-sepharose beads by standard methods. ISG15 was expressed and purified from bacteria and labeled with 32P-γ-ATP (see materials and methods). Herc5 did not express well in bacteria, and no activity was detectable when the protein was purified from insect cells (data not shown). Therefore, cell extract of Herc5-transfected 293T cells was used as a source of the E3. Cell-extract of non-transfected 293Ts was used as the negative control. As seen in Fig 3.14, a DTT resistant ISG15 conjugate corresponding to a molecular weight of 85 kDa was detected only when wild-type Herc5-transfected cell extract was included in the reaction mix. The band was not detected with cell extract of cells transfected with the C994A mutant of Herc5 or wild-type Herc6. The same conjugate was also seen when extract from IFN-β-treated cells was added to the reaction, further indicating that a bona fide substrate was being modified by ISG15 *in vitro*. I ruled out the possibility that the band corresponded to a Herc5 auto-conjugation product because cells transfected with different sized tags (TAP and Flag) gave the same conjugate (data not shown). Further experiments will be required to determine the identity of the substrate (85 kDa band) that is conjugated to ISG15 *in vitro*. While this is still a crude system, this is the first assay system to reveal Herc5-dependent ISG15 conjugation activity *in vitro*. This represents a starting point for further purification of Herc5 and other components required for ISG15 conjugation.

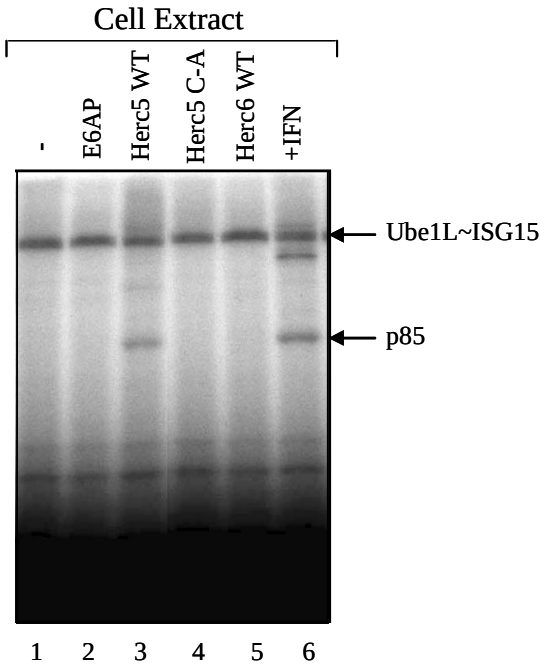


Figure 3.14 In vitro assay for ISG15 conjugation. Ube1L and UbcH8 were expressed and purified from insect cells. ISG15 was expressed and purified from bacteria and labeled with ^{32}P-γ-ATP prior to use. Exracts of 293T cells mock transfected (lane 1) or transfected with plasmids expressing E6AP (lane 2), wild type Herc5 (lane 3), the C994A mutant (lane 4) or Herc6 (lane 5) were used as a source of E3. Purified E1, E2 and labeled ISG15 were incubated in the reaction buffer at room temperature for 30 min, with cell extract. Reactions were stopped by adding 1X SDS-PAGE loading buffer without DTT. The p85 conjugate and the Ube1L~ISG15 thioester are indicated.

3.3 Discussion

The results presented here show that the Herc5 HECT E3 enzyme is required for conjugation of ISG15 to a broad range, and potentially the complete range, of natural ISG15 target proteins. The coordinate induction of ISG15, Ube1L, UbcH8, and Herc5 by IFN-β is also consistent with a central role for Herc5 in the conjugation system. The most straightforward model for the involvement of Herc5 in the ISG15 system is that it participates directly as an ISG15 ligase, as part of an ISG15 transthiolation cascade from Ube1L to UbcH8 to Herc5, with Herc5 catalyzing ISG15-target protein conjugation. This model is supported by our proteomic analysis of ISG15-conjugated proteins, where Ube1L, UbcH8, and Herc5 were all identified as ISG15-conjugated proteins (197). In addition, a HECT E3 enzyme that can interact with UbcH8 (Saccharomyces cerevisiae Rsp5) was shown to form an ISG15 thioester intermediate and catalyze ISG15 conjugation *in vitro* (196). This indicates that there are no inherent structural impediments that prevent a HECT E3s from catalyzing ISG15 conjugation.

One approach to prove that Herc5 participates directly as an ISG15 ligase would be to establish an *in vitro* system in which Herc5 forms an ISG15 thioester and conjugates ISG15 to a natural ISG15 target protein. However, I have not yet succeeded in preparing purified recombinant Herc5 protein or protein fragments (e.g. the HECT domain alone) that can be activated with either ubiquitin or ISG15 *in vitro*. Hence, mammalian cell extract containing active Herc5 was used as a source of E3s. Although a thioester bond between Herc5 and ISG15 has so far not been detected, I have

demonstrated a direct correlation between active Herc5 and an 85 kDa conjugate seen in *in vitro* assays. Future experiments in the lab will focus on further refining this assay system and determining the identity of the 85 kDa conjugate. Also, other mutants of Herc5 will be expressed and purified from insect cells with the hope that some of these might be active in an *in vitro* assay.

Alternative models for the role of Herc5 in ISG15 conjugation cannot be ruled out. For example, it is conceivable that Herc5 might catalyze a ubiquitination event that is a prerequisite for ISG15 conjugation. However, siRNA-mediated depletion of the E1Ub enzyme did not result in any decrease in Herc5-dependent ISG15 conjugation activity in non-IFN-treated cells (data not shown). In addition, Herc5 is more likely to have a direct role in ISG15 conjugation because it was auto-conjugated to ISG15 *in vivo*. A proteomics-based approach identified 158 ISG15 target proteins at a very high confidence level (197). If Herc5 acts directly as an ISG15 E3 enzyme, this would imply that this single E3 has the capacity to recognize, either directly or indirectly, a large number of target proteins. SUMO conjugation utilizes a relatively small number of E3s, however, Ubc9, the SUMO conjugating enzyme, is distinct from other conjugating enzymes in that it mediates substrate interaction. Most substrates are sumoylated on a conserved ΨKXD/E motif (where Ψ is a bulky aliphatic group and X is any amino acid), which is recognized by Ubc9 (155). Analysis of target proteins identified to date has not revealed a motif common to target proteins that might be directly recognized by Herc5. An alternative is that additional cellular proteins or factors might mediate the interaction of Herc5 with individual target proteins or subsets of target proteins. Our previous

demonstration that at least some HECT E3s have the capacity to catalyze ISG15 conjugation *in vitro* (196) raises the question of what might limit the participation of HECT E3s other than Herc5 from participating in the ISG15 conjugation system. The involvement of accessory or "licensing" factors might explain how other HECT E3s are prevented from catalyzing ISG15 conjugation.

Of the six human Herc proteins (Herc1–6), Herc6 is the most similar to Herc5 (48 % identical), and its expression is also induced by IFN-β. While I did not detect an effect of Herc6 siRNAs on overall ISG15 conjugation, it is possible that Herc6 plays a minor role in ISG15 conjugation, perhaps targeting a limited set of proteins compared with Herc5. Further biochemical comparisons of Herc5 and Herc6 will be important for identifying the determinants of Herc5 that confer its dominant function in the ISG15 system. In chapter 4, I describe the results of Herc5-Herc6 chimeric proteins and also show that Herc6 has the capability to function with ISG15.

In addition to Herc5 and Herc6, our microarray analysis identified several TRIM (tripartite motif) proteins that were induced by IFN-β, some of which have been shown to function as ubiquitin E3s (122) and at least one of which (TRIM25/Efp) has been reported to interact with UbcH8 (175). siRNAs that targeted TRIM25/Efp had no effect on the overall pattern or accumulation of ISG15 conjugates nor did transfection of TRIM25 expression vectors boost or alter the overall pattern of ISG15 conjugates. A recent report showed that Efp could auto-conjugate ISG15, however, the levels of ubiquitin-conjugated Efp were much higher than that of ISG15 conjugated Efp (134),

indicating that it functions more efficiently with ubiquitin. Another report stated that Efp/TRIM25 could conjugate ISG15 to 14-3-3σ (200). Efp is a well characterized ubiquitin ligase implicated in breast cancer because it targets 14-3-3σ, a negative regulator of cell cycle, for proteolytic degradation. The finding that it also conjugates ISG15 to the same substrate 14-3-3σ, could be because in interferon-treated cells, the high levels of UbcH8, ISG15 and Efp/TRIM25, might force the ligase to react with ISG15, albeit less efficiently. The authors propose that in the absence of interferon-induced Ube1L, UbcH8 and ISG15, Efp/TRIM25 functions as a ubiquitin ligase, but it might perform a dual role of a ubquitin/ISG15 ligase in the presence of ISG15 conjugating enzymes. Recently, Gack et al showed that Efp/TRIM25 was essential for RIG-I mediated anti-viral activity (35). Efp/TRIM25 mediates robust ubiquitination of the caspase recruitment domains (CARDS) of RIG-I, which enhances RIG-I downstream signaling. This role of Efp/TRIM25 might explain why a ubiquitin ligase is upregulated in the presence of interferons.

The demonstration that Herc5 is required for ISG15 conjugation to a broad spectrum of target proteins in human cells will facilitate analysis of effects of ISG15 conjugation on target proteins and elucidation of the role of ISG15 conjugation in anti-viral and anti-microbial responses. In chapter 5, I will describe the identification of an additional cellular protein that is required for Herc5-dependent ISG15 conjugation.

CHAPTER 4: ANALYSIS OF HERC5 MUTANTS AND HERC5-HERC6 CHIMERAS

4.1 Introduction

Herc5 belongs to the HECT and RCC1-like domain (HERC) family of proteins. In humans, this family is characterized by six members, Herc1-6, all of which have one or more RCC1 (Regulator of chromosome condensation)-like domains (RLDs) at the N-terminus and a HECT domain at the C-terminus (59). The HERC family is distinctly divided into large and small members. Herc1 and Herc2, which are about 500 kDa, belong to the former category whereas Herc3-6 are about 120 kDa and are part of the latter group.

Herc5 contains one RLD from residues 157 through 377, which is composed of four RCC1-like repeats of approximately 50 amino acids each. These repeats were first discovered in the RCC1 protein, which functions as a guanine nucleotide exchange factor (GEF) for the small G-protein Ran. RCC1 consists solely of seven tandem repeats, which form a seven-bladed β-propeller structure, one face of which interacts with Ran (154) and the other side binds chromatin histones H2A and/or H2B (136). The RLDs of Herc1 and RPGR (Retinitis Pigmentosa GTPase Regulator) function as protein interaction domains, binding clathrin heavy chain and PDE-δ (cyclic GMP phosphor-diesterase delta subunit), respectively (109, 157). The function of the RLDs of other Herc proteins is not known, but it is generally thought that they are protein-protein interaction motifs that might be

important for recruiting substrates and/or regulation of the catalytic activity of Herc proteins. Mutants of Herc5 were made to test the importance of the RCC1-like repeats for Herc5 activity.

Herc5 has a HECT domain spanning the last 350 amino acids of the protein. Prior to the finding described in the previous chapter, HECT domain proteins were thought to mediate only ubiquitin conjugation. Herc5 is the first example of a HECT E3 that catalyzes the transfer of a Ubl. A mutation of the active site cysteine resulted in an inactive enzyme, implying that Herc5 catalyzes ISG15 conjugation via an ISG15 thioester intermediate (18). As mentioned in Chapter 1, a highly conserved phenylalanine usually at the fourth to last position from the C-terminus of HECT domains (-4F) was shown to be important for transferring ubiquitin to the substrate without affecting thio-ester bond formation (161). It was proposed that the -4F might be important for correctly orienting the ubiquitin while it is tethered via a thioester bond to the active site cysteine, allowing the substrate lysine to attack. In Herc5, this phenylalanine is at the second to last position (-2F) and analysis of a mutant at this position will be described below.

Pfam analysis revealed that in addition to the RLD and HECT domains, the central region of Herc5 (residues 554 and 579) harbors a domain with weak similarity to a Death Domain (DD). Death domains were first identified in tumor necrosis factor receptor (TNFR1) and FAS (172), where they cause aggregation of their respective receptors through homotypic interactions. Initially, death domain bearing proteins were thought to be involved in apoptotic pathways only. Subsequently, these domains have been discovered in other signaling molecules also and are considered to be protein

interaction motifs. Given the low similarity to characterized DDs, it is unlikely that this domain operates as a true DD. However, we were intrigued by the position of this domain within Herc5, which corresponds closely to substrate binding domains in some other HECT E3s. For example, the E6-binding site of E6AP is centered approximately 100 residues upstream of the HECT domain, spanning 17 amino acids (72). The third WW domain of Rsp5, which is required for binding to several substrates, is also centered approximately 100 amino acids upstream of the HECT domain (30, 64) (See Fig 4.1). The death-like domain is similarly positioned, approximately 100 residues upstream of the Herc5 HECT domain, and could potentially be involved in mediating substrate recognition. In light of the previous results with HECT proteins, we will refer to this 25 amino acid region as the putative substrate binding box (SBB). The effects of deletions within this region will be describe

Among the HERC family members, Herc6 is most closely related to Herc5, with the two proteins sharing 48 % overall identity, and 66% identity within the HECT domain. Herc6 has five RCC1-like repeats that extend from residues 42 to 305, as compared to the four RCC1-like repeats found in Herc5 (Fig 4.2). Sequence analysis of Herc6 did not show any conserved motifs in the region corresponding to the substrate binding box (SBB) of other HECT proteins, but secondary structure prediction revealed that both Herc5 and Herc6 share a similar structure throughout the length of the protein, including the SBB. Further, the Herc6 gene, like Herc5, is also transcriptionally induced by interferons α and β (18). Although my previous data from human cell lines indicates that Herc6 is not a major ligase for ISG15, I tested whether it is conjugated to ISG15. I

show here that Herc6 is a substrate for Herc5-dependent ISG15 conjugation, and it also exhibits auto-conjugation of ISG15. Further, chimeras were made between Herc5 and Herc6 to determine which regions of the protein confer the ability to conjugate ISG15 to substrates.

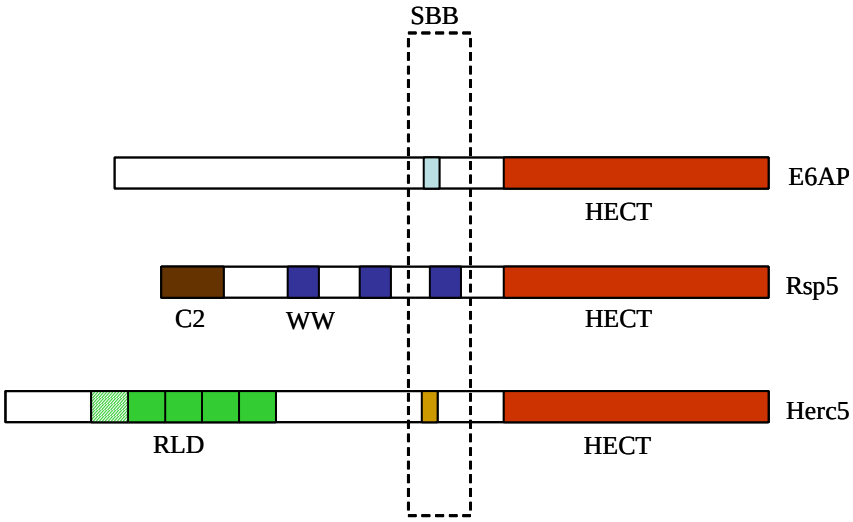


Figure 4.1 A schematic showing the substrate binding box (SBB). The schematic shows E6AP, Rsp5 and Herc5 and their respective domains. The SBB is positioned approximately 100 amino acids upstream of the HECT domain and is marked by a dotted rectangle in the figure. It corresponds to the E6-binding region of E6AP and the 3rd WW-domain of Rsp5 that binds to at least a subset of targets. This might also be the region of Herc5 that is important for mediating substrate interaction.

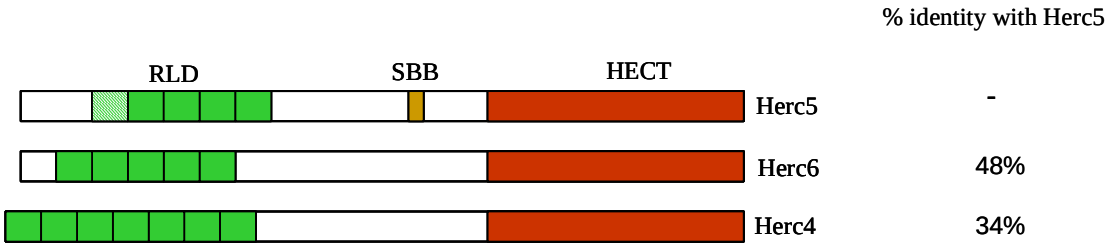


Figure 4.2 A schematic comparing Her4, Herc5 and Herc6. The RCC1-like domains (RLD) and the HECT domains of the Herc proteins are shown in green and maroon, respectively. Each RCC1-like repeat is represented by one square. Herc5 has a putative fifth RCC1-like repeat, indicated by the striped green square. In addition to sharing close sequence similarity with Herc5, the secondary structure of Herc6 is also predicted to be similar to that of Herc5. Like Ube1L, UbcH8, Herc5 and ISG15, the gene for Herc6 is transcriptionally induced by IFN-β.

4.2 Results

Analysis of ΔF mutant of Herc5

While HECT domains have been well characterized with respect to ubiquitin catalysis, Herc5 is the first example of a HECT domain protein involved in Ubl conjugation. To determine whether the conserved phenylalanine residue, which is at the second to last position in Herc5 (-2F), affects transfer of ISG15 to substrates, the last two amino acids of Herc5 were deleted to create the ΔF mutant. This mutant was tested in HeLa cell transfections for its ability to support ISG15 conjugation. It was shown previously that non-interferon-treated cells transfected with plasmids expressing ISG15, Ube1L, and UbcH8 showed a low level of ISG15 conjugation which increased dramatically when wild type Herc5 was included in the transfection. In contrast, the catalytically inactive C994A mutant did not increase the conjugates beyond the basal level. This assay was used to test the ΔF mutant. Figure 4.3 shows a higher level of ISG15 conjugates accumulated when cells were transfected with wild type Herc5 (lane 2) as compared to cells with no transfected Herc5 (lane 1) or the C994A mutant (lane 3). When cells were transfected with the ΔF Herc5 mutant along with E1, E2 and ISG15, there was no boost in ISG15 conjugates (lane 4), indicating that the ΔF mutant could not conjugate substrates. Auto-conjugation is a property of some ubiquitin ligases and I had previously shown that Herc5 could ligate ISG15 to itself. TAP-tagged Herc5 was transfected with the ISG15 conjugation machinery, and extracts were probed with anti-TAP antibody to detect auto-conjugation. Wild type Herc5 showed strong auto-conjugation, as detected from the smear of high molecular weight conjugates, whereas

the C994A mutant was completely inactive and showed no conjugates (Fig 4.2 lanes 5 and 6). In comparison, the ΔF mutant showed very few conjugates and a large proportion of the protein was not modified, clearly indicating that this mutant was defective in auto-conjugation (Fig 4.2 lane 7). Similar results were obtained with the -4F mutants of E6AP and Rsp5, which were defective in transferring ubiquitin onto substrates (161). While it is not known whether ΔF is capable of forming a thioester bond with ISG15, based on the results of analogous -4F mutants in other HECT E3s it is expected that the thioester bond will not be affected.

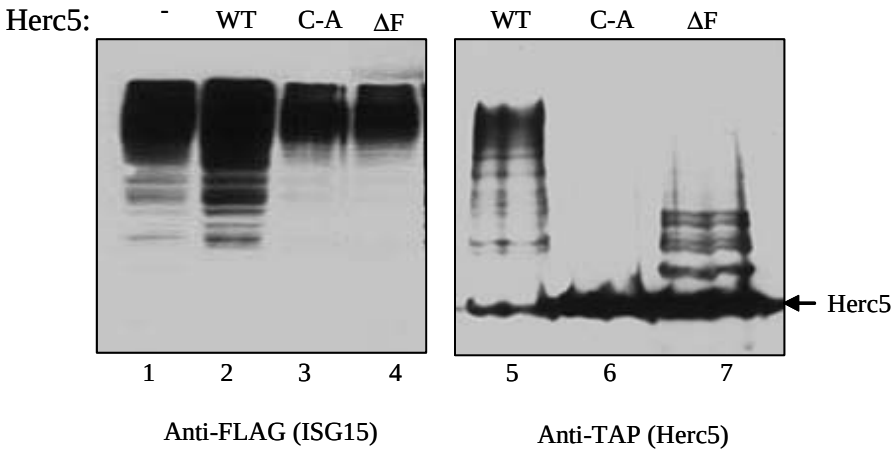


Figure 4.3 The ΔF mutant, similar to -4F mutants of ubiquitin ligases, is defective in auto-conjugation. HeLa cells were transfected with Ube1L, UbcH8 and FLAG-ISG15, with no Herc5 (lane 1), with wild type Herc5 (lanes 2 and 5), with C994A mutant (lanes 3 and 6) or with the ΔF mutant. Cell extracts were immunoblotted with Anti-FLAG antibody (left panel) or with anti-TAP antibody (right panel).

Analysis of RLD and SBB mutants

As mentioned above, Herc5 has four RCC1-like repeats spanning residues 157 to 377, which are postulated to be protein interaction motifs. To assess the importance of these repeats in ISG15 conjugation, a set of truncations were made (Fig 4.4). Substrate conjugation as well as auto conjugation activities of each mutant were tested in HeLa cell transfections. The ΔRCC construct, spanning residues 381-1024, lacked the RCC1-like repeats. This mutant was unable to conjugate ISG15 to substrates, but it retained auto-conjugation activity (Fig 4.5). This suggests that the RCC1-like domains are involved in substrate recognition. It is interesting to note that the auto-conjugation pattern of this mutant was different from that of the wild type protein. While the wild type protein showed distinct ISG15 conjugates, the ΔRCC mutant showed a smear of very high molecular weight conjugates (Fig 4.5 compare lanes 7 and 9). Also, a bigger fraction of the protein pool was conjugated in the ΔRCC mutant compared to the wild type protein.

Figure 4.4 Schematic showing the RLD mutants and the SBB mutant. The RLD, the SBB and HECT domains are shown in green, brown and red, respectively.

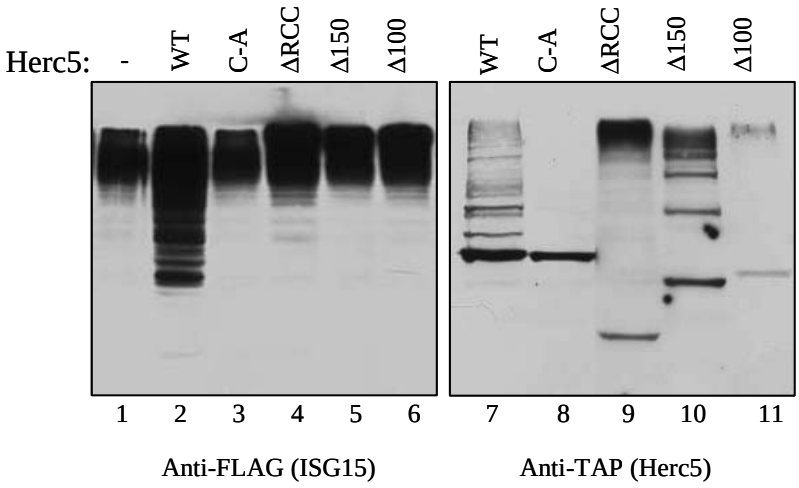


Figure 4.5 Auto-conjugation and substrate conjugation activities of the RLD-mutants. HeLa cells were transfected with Ube1L, UbcH8 and FLAG-ISG15 with no Herc5 (lane 1) or wild type Herc5 (lane 2 and 7) or with Herc5 mutants as indicated. Cell extracts were analyzed by immunoblotting with anti-FLAG antibody (left panel) or anti-TAP antibody (right panel).

An N-terminal truncation deleting the first 156 residues of the protein (Δ150) retained all the RCC1-like repeats and it was analyzed for both substrate modification as well as auto-conjugation. The auto-conjugation activity of this mutant was intact, and it resembled the ΔRCC mutant in that a major portion of the protein was conjugated to ISG15 (Fig 4.5 lane 10). However, this mutant did not increase the levels of ISG15 conjugates when co-transfected into cells with E1, E2 and ISG15, indicating that it was unable to modify substrates (Fig 4.5 lane 5). This implies that the first 150 amino acids of the protein are important for substrate conjugation. However, there is a fifth RCC1 repeat with a low match score (Pfam) between residues 130 and 153, which might be contributing to substrate conjugation. The first 100 amino acids of Herc5 were deleted (the Δ100 mutant), leaving this additional putative RCC1 repeat intact. As with the previous two N-terminal truncations, this mutant also displayed efficient auto-conjugation but no substrate conjugation (Fig 4.5 lanes 6 and 11). This clearly indicates that the first 100 amino acids of Herc5 are required for substrate conjugation. Although no recognizable domains are present in this stretch of the protein, it is possible that the region up to the first 100 amino acids might contain determinants important for substrate conjugation. Alternatively, it is possible that the N-terminus of the protein is needed for proper folding of the RCC1-like repeats, which are responsible for mediating substrate interaction. Future work in the lab will test these various hypotheses.

To determine the importance of the substrate binding box (SBB) for Herc5-mediated ISG15 conjugation, a mutant protein lacking this domain (amino acids 554-579) was constructed (ΔSBB). This mutant did not conjugate ISG15 to substrates, implying

that the SBB is important for mediating interactions with substrates, either directly or via other factors or adaptors that recruit substrates. Like the N-terminal mutants described above, the ΔSBB mutant was very efficiently auto-conjugated to ISG15 (Fig 4.6).

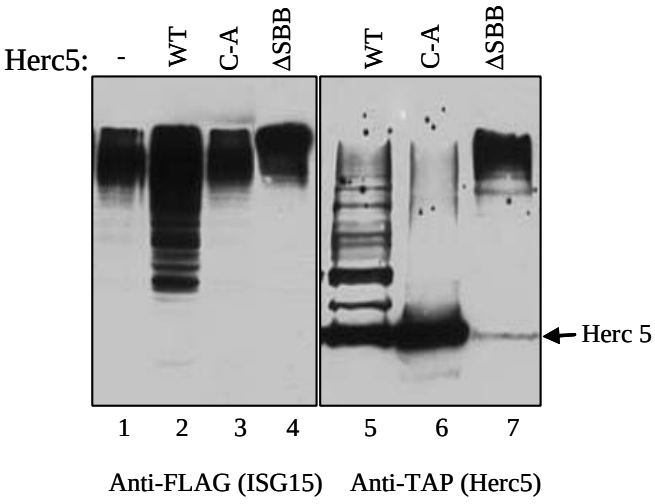


Figure 4.6 Auto-conjugation and substrate conjugation activities of ΔSBB mutant. HeLa cells were transfected with plasmids expressing Ube1L, UbcH8 and ISG15, with no Herc5 (lane 1) or wild type Herc5 (lanes 2 and 5) or the C994A mutant (lanes 3 and 6) or the ΔSBB mutant (lanes 4 and 7). Cell extracts were transferred onto a nitrocellulose membrane and probed with anti-FLAG antibody (left panel) or Anti-TAP antibody (right panel).

Herc6 is conjugated to ISG15

As mentioned in the introduction, among the HERC members, Herc6 is most closely related to Herc5 (48% overall identity) and the two proteins share similarities in sequence and secondary structure predictions. Further, like Herc5 and the other enzymes of the ISG15 pathway, Herc6 expression is induced by IFN-β (18) suggesting that Herc6 might be a part of the conjugation machinery. My earlier results with siRNAs showed that knocking down Herc6 did not have an effect on ISG15 conjugation in interferon-treated cells (18). Similarly, transfecting Herc6 plasmid did not boost conjugates in non-interferon-treated cells co-transfected with Ube1L, UbcH8 and ISG15. These results indicated that Herc6 does not play a major role in ISG15 conjugation, however we could not rule out that Herc6 might play a minor role, perhaps by targeting a small number of specific substrates.

An alternative assay for whether Herc6 might be involved in ISG15 conjugation was to determine whether Herc6 catalyzed auto-conjugation to ISG15. HeLa cells were transfected with Ube1L, UbcH8, ISG15 and TAP-tagged Herc6, and an immunoblot was probed with anti-TAP antibody to detect Herc6. Figure 4.7A shows high molecular weight conjugates of Herc6 only in presence of Ube1L, UbcH8, and ISG15. No conjugates were detected when either UbcH8 or ISG15 were omitted from the transfection mix. To determine whether these ISG15 conjugates were a result of Herc6 auto-conjugation, the catalytic cysteine residue at position 985 was mutated. When the C985A mutant was transfected into cells along with the E1, E2 and ISG15, conjugation

was decreased but not eliminated (Fig 4.7 B, compare lanes 1 and 3), indicating that at least some of the Herc6 conjugates were not a result of auto-conjugation.

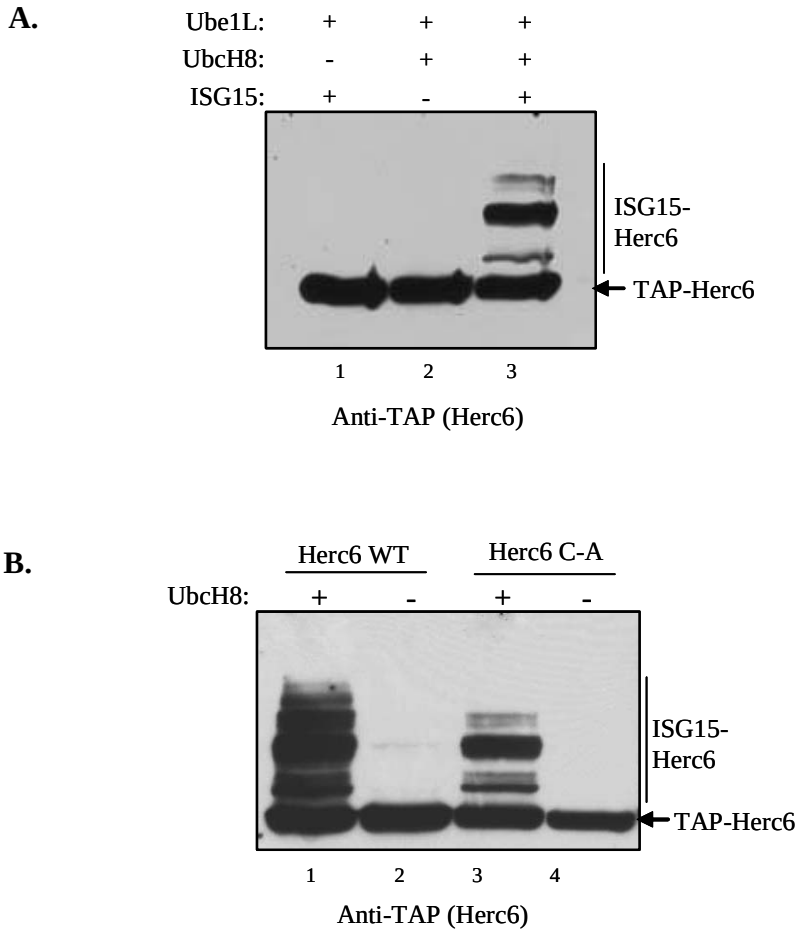


Figure 4.7 Herc6 is conjugated to ISG15 in the presence of Ube1L and UbcH8. A) HeLa cells were co-transfected with TAP-Herc6 plasmid and various combinations of plasmids expressing Ube1L, UbcH8 and ISG15. Cell extracts were analyzed by immunoblotting with anti-TAP antibody. B) Ube1L, ISG15 and Herc6 (wild type or the C985A mutant) were co-transfected with (lanes 1 and 3) or without (lanes 2 and 4) UbcH8. Cell extracts were analyzed by immunoblotting with anti-TAP antibody.

I had previously shown that there is a basal level of Herc5 in non-IFN-treated cells, which is responsible for the ISG15 conjugates that accumulate in a triple transfection of plasmids expressing Ube1L, UbcH8 and ISG15 (18). To determine whether Herc6 conjugation was Herc5-dependent, cells were first treated with siRNAs to knock-down Herc5 and then co-transfected with Ube1L, UbcH8 and ISG15 and TAP-tagged Herc6. Figure 4.8 shows that in the case of Herc6 C985A mutant, knocking down Herc5 abolished all ISG15 conjugates (lane 4), indicating that the C985A mutant was conjugated by Herc5. On the other hand, wild type Herc6 retained some conjugates even when Herc5 was knocked down (Fig 4.8 lane 2). Since this conjugation is not dependent on Herc5 and is dependent on the catalytic activity of Herc6, it is likely the result of ISG15 auto-conjugation activity of Herc6.

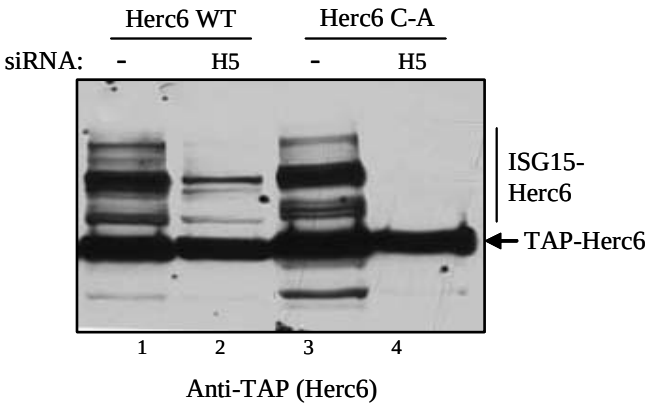


Figure 4.8 Herc6-ISG15 conjugation is a combination of auto-conjugation and Herc5-dependent conjugation. HeLa cells were mock-transfected (lanes 1 and 3) or transfected with siRNAs against Herc5 (lanes 2 and 4) and subsequently transfected with plasmids expressing Ube1L, UbcH8, ISG15 and wild type Herc6 or the C985A mutant. Cell extracts were immunoblotted with anti-TAP antibody.

To further investigate the significance of Herc5 and Herc6 auto-conjugation, I determined whether another HERC protein, Herc4, has ISG15 auto-conjugation activity. Herc4 is 34% identical to Herc5 and phylogenetic analyses indicate that Herc4 is likely to be the common ancestor of all HERC proteins (59). Herc4 has four RCC1-like repeats, spanning residues 7 to 230, and unlike Herc5, Herc4 does not have a significant leader sequence preceding the RLD (Fig 4.2). There is no identifiable motif present in the region corresponding to the Herc5 SBB and secondary structure prediction (network protein sequence analysis – NPSA) indicates that Herc4 is significantly different from Herc5 over this stretch. Also, as our microarray results indicate, the gene for Herc4 is not upregulated by IFN-β. Figure 4.9 shows that TAP-Herc4 was not conjugated to ISG15 under the same conditions where TAP-Herc5 and TAP-Herc6 were both efficiently modified. As an additional negative control, TAP-E6AP also did not auto-conjugate ISG15 (data not shown), strongly suggesting that the ability to function with ISG15 *in vivo* is limited to Herc5 and Herc6.

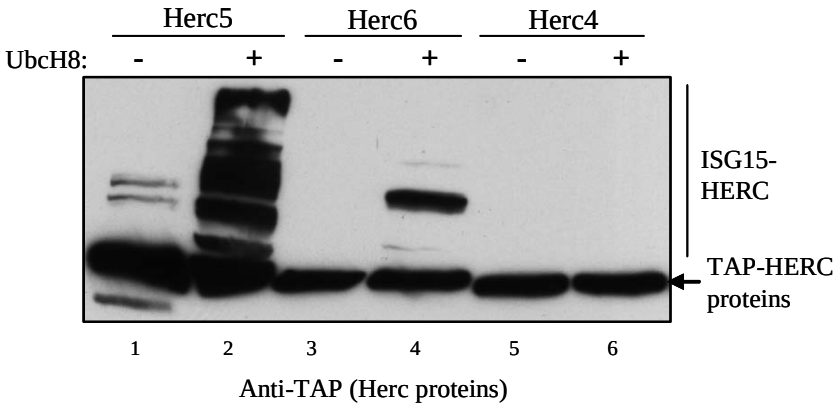


Figure 4.9 The ability to function with ISG15 is limited to Herc5 and Herc6. HeLa cells were transfected with plasmids expressing Ube1L, UbcH8, ISG15 and TAP-Herc5 (lane 2) or TAP-Herc6 (lane 4) or TAP-Herc4 (lane 6). As a control, each of the Herc proteins were also transfected without UbcH8 expressing plasmids (lanes 1, 3 and 5). Cell extracts were transferred to a nitro-cellulose membrane and probed with anti-TAP antibody.

Herc5-Herc6 chimeras

In spite of the similarities between Herc5 and Herc6, Herc5 is the only E3 that has been found to have a broad effect on ISG15 conjugation to substrate proteins. To investigate whether this disparity mapped to the HECT domain or the region upstream of the HECT domain, chimeric proteins were expressed where the HECT domains of the two proteins were switched (Fig 4.10). The chimera with the N-terminus of Herc5 and HECT domain from Herc6 was referred to as Herc 5/6 and the converse chimera was labeled Herc 6/5. Both the chimeric proteins were transfected into non-interferon-treated HeLa cells to check whether they could catalyze auto-conjugation and enhance conjugation to substrates. Neither chimera increased the level of ISG15 conjugates over that seen in the absence of any Herc5 protein (Fig 4.11 A), indicating that the HECT domain of Herc6 cannot substitute for the Herc5 HECT domain. This could be because the Herc6 HECT does not function as efficiently with UbcH8 and/or ISG15, as suggested from the respective auto-conjugation activities of Herc5 and Herc6 (Fig 4.9). It is also clear that other determinants within Herc5 (*e.g.* RLDs or SBB) are important for substrate conjugation, since the Herc5 HECT domain was not sufficient to convert Herc6 into a major ISG15 ligase. It should be mentioned here that we have had little success in our lab getting active chimeras by exchanging HECT domains of various proteins (*e.g.* Rsp5 and E6AP). This suggests that there may be interactions between the N- and C-terminal regions of HECT E3s and switching one HECT domain for another might disrupt their function. Both the chimeras displayed auto-conjugation activity, however, there was a difference in their patterns of auto-conjugation (Fig 4.11 B). Chimera Herc6/5 was

modified similar to the ΔRCC mutant, with very high molecular weight conjugates at the top of the gel (Fig 4.11 B compare lane 3 with lanes 4 and 5). One possible model that could explain this difference is that the N-terminal region of Herc5 has an inhibitory effect on protein activity and any mutants that delete/replace this region display an increased auto-conjugation activity (Also, see discussion).

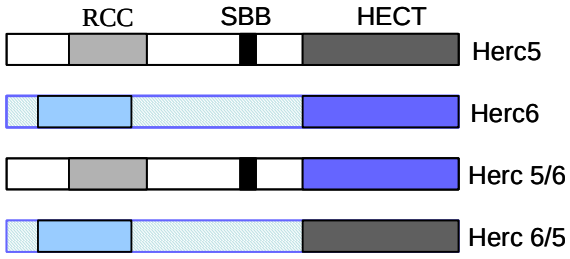


Figure 4.10 Schematic showing Herc5-Herc6 chimeras. The Herc5 and Herc6 proteins are shown in black and blue, respectively. Herc 5/6 retains the N-terminus of Herc5 while the HECT domain belongs to Herc6. Herc 6/5 is the converse chimera with the Herc6 N-terminus and the Herc5 HECT domain.

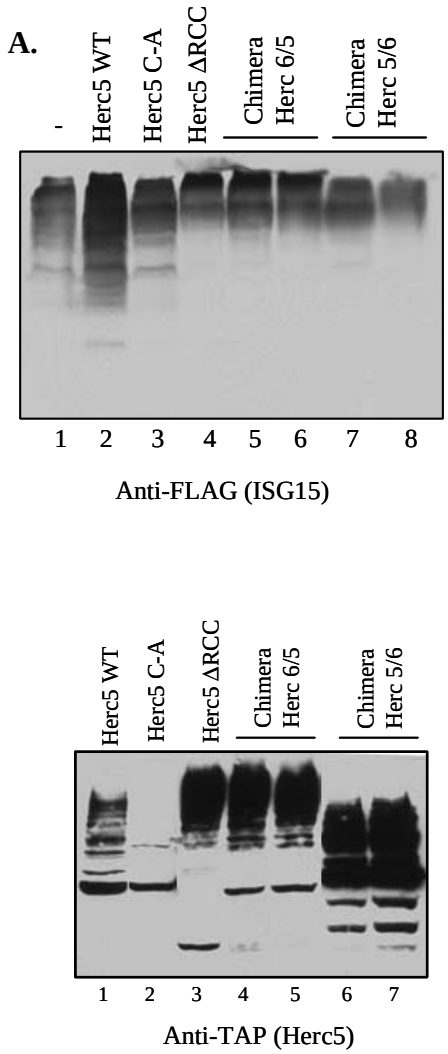


Figure 4.11 Substrate conjugation and auto-conjugation activities of Herc5-Herc6 chimeras. HeLa cells were transfected with plasmids expressing Ube1L, UbcH8, ISG15 and different Herc5 constructs, as indicated. Cell extracts were prepared and analyzed by immunoblotting with A) anti-FLAG antibody to detect ISG15 conjugates and B) anti-TAP antibody to detect Herc5.

While the HECT domains of Herc5 and Herc6 represent the most conserved region of the two proteins, they differ in the position of the conserved phenylalanine residue found at the fourth position from the carboxyl terminus of most HECT E3s (-4F). This residue was previously shown to play a role in promoting ubiquitin conjugation (161). In Herc5, this phenylalanine is at the second from last position (-2F), while in Herc6 it is at the ninth from last position (-9F). We tested whether this difference might be related to the ability of Herc5 to function in the ISG15 pathway by replacing the last residue of Herc5 (FG) with the last eight residues of Herc6 (FVSPMLTGS). Fig 4.12 shows that this mutant (C5–6) stimulated ISG15 conjugation as efficiently as wild-type Herc5. The divergent carboxyl-terminal tails are therefore not the basis for the functional difference between Herc5 and Herc6 in the ISG15 system.

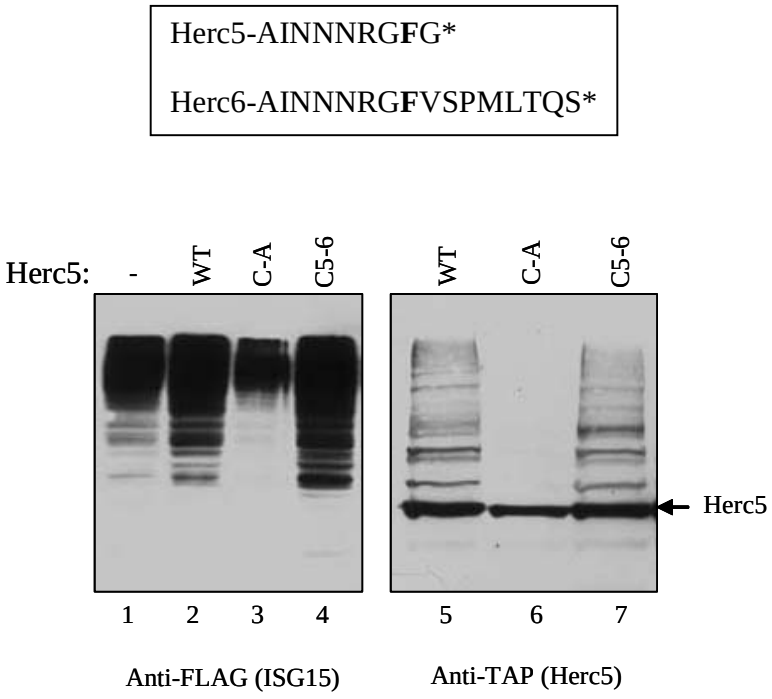


Figure 4.12 Auto-conjugation and substrate conjugation activities of the C5-6 chimera. HeLa cells were transfected with Ube1L, UbcH8 and FLAG-ISG15, with no Herc5 (lane 1), with wild type Herc5 (lanes 2 and 5), with the C994A mutant (lanes 3 and 6) or with the C5-6 chimera. Cell extracts were immunoblotted with Anti-FLAG antibody (left panel) or with anti-TAP antibody (right panel). The box shows the sequence of the C-terminal residues of Herc5 and Herc6. The residues following the Phe were switched between the two proteins.

4.3 Discussion

A set of point mutations and deletions were made to investigate the role of each of the Herc5 domains: RCC1-like repeats, the putative substrate binding box (SSB) and the HECT domain. While it is known that the HECT domain is responsible for the catalytic activity of the protein, this is the first example of a HECT domain involved in non-ubiquitin catalysis and it was not known whether the mechanisms would be conserved. The residues that are important for ubiquitin thioester bond formation (catalytic cysteine) and for transfer of ubiquitin to substrates (-2F) were shown here to be important for ISG15 catalysis, suggesting that ISG15 conjugation employs a similar mechanism. Currently, the *in vitro* thioester assay does not work with Herc5, but our work with other HECT E3s has shown that this phenylalanine residue does not interfere with thio-ester bond formation.

The role of RCC1-like repeats was analyzed by making a set of N-terminal truncations. So far, it is not clear whether the RCC repeats are important for ISG15 conjugation to substrates, but a region N-terminus to the RCC1-like repeats was required for conjugating ISG15 to substrates. An inspection of the residues upstream of the RCC1-like repeats did not show any known motifs. While it is possible that these residues form a hitherto unknown motif important for binding proteins, it could also be that this region is required for the correct folding of the RCC1-like repeats. In other proteins RLDs are known to represent protein-protein interaction motifs (109, 157) and here, too, they maybe required for recruiting substrates and/or adaptor protein(s). The following chapter will describe the identification of one such adaptor protein.

Interestingly, N-terminal truncations and the ΔSBB mutant were auto-conjugated more robustly than wild type Herc5 (Fig 4.5 and 4.6). While only a relatively small fraction of Herc5 was auto-conjugated to ISG15 in the presence of the E1, E2 and E3, almost the entire pool of the ΔRCC1 and ΔSBB proteins were modified under the same conditions. A similar increase in activity was seen in the Herc6/5 chimera, where the N-terminus of Herc5 had been replaced by the N-terminus of Herc6. One model to account for these observations is the Herc5 normally exists in a closed and catalytically inactive conformation, possibly mediated by interactions between the RLD and the SSB domains. Deletion of either of these domains results in a constitutively activated enzyme (hence the robust auto-conjugation activity), however the fact that normal substrates are not recognized by either the ΔRCC1 or ΔSBB mutants indicates that both regions are critical for interacting with substrates and/or adaptor proteins.

The induction of Herc6 by interferon and its similarity to Herc5 made it a likely candidate for a ligase of ISG15. Even though our earlier studies showed that knocking down Herc6 did not have an obvious effect on overall substrate conjugation (18), we had postulated that Herc6 might be a minor ligase for this pathway. The results presented here show that Herc6 is a substrate of Herc5-mediated ISG15 conjugation and it could also auto-conjugate ISG15, though not as robustly as Herc5. Both Herc5 and Herc6 are located on chromosome 4, and Herc5 was proposed to have been derived from Herc6 via gene duplication (59). Rodents (rats, mice) have a homolog for Herc6 but not a Herc5 homolog. This raises the question of how mice conjugate ISG15 to substrates (42). Also, it is not clear whether the gene duplication event did not occur in rodents or whether they

lost the Herc5 gene during evolution. The latter is a more likely possibility, since the genomes of other mammals (dog, cow) retain a Herc5-encoding gene and suggest that the gene duplication event giving rise to Herc5 occurred before these families split from a common ancestor. It has been reported that mice have a gene for Ube1L and UbcM8, both of which are similar to their human homologs (96). I showed that Herc6 can function with Ube1L and UbcH8 to auto-conjugate ISG15. In light of the above results, it is conceivable that Herc6 plays a major role in ISG15 conjugation in rodents.

CHAPTER 5: IQGAP1 IS REQUIRED FOR ISG15 CONJUGATION

5.1 Introduction

The identification of Herc5 as the major E3 for ISG15 raised several important questions. First, how is it possible for a single enzyme to recognize such a diverse spectrum of substrates? There may be consensus sites on substrates that are targeted by the E3, however, there have been no obvious motifs by sequence comparison. Alternatively, there might be accessory factor(s) that bind Herc5 and mediate its interaction with target proteins. Second, what prevents other HECT E3s such as E6AP, which are capable of interacting with UbcH8 and forming a thioester bond with ISG15 *in vitro*, from functioning with ISG15 *in vivo*? This could be explained if a protein was exclusively binding enzymes of the ISG15 pathway, thus channeling their activities and also preventing proteins of the ubiquitin system from impinging. Such proteins, called scaffolding proteins, are known to exist for other signaling pathways such as the MAP kinase pathway. There is considerable diversity in the MAP kinase pathway, not just with regard to stimuli, but also downstream targets. In *S. cerevisiae* there are as many as six MAP kinase cascades that respond to various external stimuli. The complexity of this pathway is further increased by the fact that some components are very similar and some of these kinases can function in more than one cascade. In order to ensure the correct

response to a particular stimulus, the proper kinase must be activated and it must phosphorylate the right targets. This specificity is maintained, at least in part, by scaffold proteins that mediate kinase-kinase interactions or kinase-substrate interactions. The SH2 and SH3 domain containing proteins are adaptors that connect receptor tyrosine kinases to their downstream targets like Ras (143). Accessory proteins can co-ordinate protein complexes and channel the signal into a particular cascade. The *S. cerevisiae* protein Ste5 was shown to interact with Ste11 (a MEK kinase - MEKK), Ste7 (a MAP or ERK kinase - MEK) and Fus3 (a mitogen activated protein kinase - MAPK) in the mating pathway (25). In some cases, scaffold proteins have also been shown to activate their binding partners. For example, the MEK kinase 1 (MEKK1) has a large N-terminal domain that interacts with different components of the JNK module and its kinase domain activates the JNK (c-Jun N-terminal kinase) pathway (129).

Drawing parallels with the MAPK pathway, an accessory protein that insulates the ISG15-specific proteins from the ubiquitin enzymes and/or co-ordinates Herc5 activity and its interaction with substrates would explain the questions raised above with regards to the role of Herc5. In a proteomics study to determine the substrates of ISG15 conjugation, IQGAP1, a scaffold protein, was identified as one of the high-confidence targets, with eleven peptides of this protein identified by mass-spectrometry (197). In previous such analyses with ubiquitin and SUMO (21, 145), enzymes that function in the Ubl pathway were found to be modified by the Ubl and similarly, Ube1L, UbcH8 and Herc5 were all conjugated to ISG15 (197). I hypothesized that IQGAP1 might be involved in the ISG15 pathway as a substrate-recruiting adaptor protein due to its multi-

domain nature (described below), and that its conjugation to ISG15 might be a reflection of its involvement in the conjugation system. Further, it might function as a scaffold that interacts exclusively with enzymes of the ISG15 pathway, thus preventing ubiquitin E2s and E3 from being mis-charged with ISG15.

IQGAP1 is a 189 kDa modular protein, which is involved in various pathways related to cell motility and cell-cell adhesion (12). There are several domains throughout the length of this protein (Fig 5.1), including a calponin-homology domain (CHD) at its N-terminus, a coiled coil region, four tandem IQ repeats, a WW domain and at its C-terminus, there is a Ras-GAP-related domain (GRD). It has been shown to interact with several cellular proteins, including several simultaneously. Complexes of IQGAP1 with Cdc42 and actin (27), with Rac1/Cdc42 and CLIP-170 (34) and with calmodulin and Cdc42 (58) have been isolated, implying that IQGAP1 might be functioning as a scaffold protein within a cell. IQGAP1 was also shown to interact with members of the MAP-kinase pathway, specifically extracellular signal-regulated kinase 2 (ERK2) and its upstream kinases MEK1, MEK2 and B-Raf (153, 158, 159). Raising or lowering the levels of IQGAP1 inhibited epidermal growth factor (EGF) mediated activation of MEK and ERK. EGF-mediated activation of B-Raf was absent in IQGAP1-null cells and binding to IQGAP1 stimulated the activity of B-Raf *in vitro* (153). It was concluded that IQGAP1 functions as a scaffold for the MAP kinase pathway, increasing the local concentrations of enzymes within the pathway, thus bringing selectivity and specificity to this complex MAP-kinase pathway.

Here I confirm that IQGAP1 is indeed modified by ISG15, as predicted from the proteomics study. siRNAs targeting IQGAP1 decreased ISG15 conjugation in IFN-β-treated cells as well as in cells transfected with Ube1L, UbcH8, ISG15 and Herc5. Binding studies showed that IQGAP1 interacts with Herc5 *in vitro* as well as *in vivo*, via the C-terminus of IQGAP1 and the amino-terminus of Herc5. Auto-conjugation of wild type Herc5, but not the ΔRCC1 or ΔSBB mutants, was lost when IQGAP1 was depleted. Together, these results indicate that IQGAP1 is an important player in the ISG15 conjugation pathway and suggest a model wherein IQGAP1 is required, at least in part, for Herc5 activity.

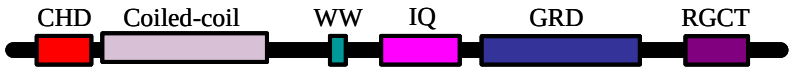


Figure 5.1 A schematic showing the domain architecture of IQGAP1.
Abbreviations: CHD - calponin homology domain; WW - a WW domain with two conserved Trp residues; IQ - IQ motif that binds calmodulin; GRD - RasGAP-related domain; RGCT - RasGAP C-terminus.

5.2 Results

IQGAP1 is conjugated to ISG15 in vivo

To confirm the result from the proteomics study that had identified IQGAP1 as a substrate of ISG15 conjugation (197), extracts from IFN-β treated cells were immunoblotted with anti-IQGAP1 antibody. Non-IFN-β-treated extracts showed a single major band of 189 kDa corresponding to endogenous IQGAP1, while extracts from IFN-β treated cells had an additional higher molecular weight band corresponding in size to a single ISG15 conjugate (Fig 5.2A, compare lanes 1 and 2). There was no difference in levels of IQGAP1 protein between the non-interferon treated sample and the interferon-treated sample, indicating that IQGAP1 is not induced by interferon. Since a majority of ISG15 conjugates are Herc5-dependent, we tested whether conjugation of IQGAP1 was also mediated by Herc5. Cells treated with Herc5 siRNAs did not show the IQGAP1-ISG15 conjugate (Fig 5.2A, lane3), whereas a control siRNA had no effect (Fig 5.2A lane 4). To confirm this result in non-IFN-β-treated cells, 293T cells were transfected with TAP-tagged IQGAP1 in the presence of plasmids expressing Ube1L, UbcH8, ISG15 and Herc5. As seen in figure 5.2B, the conjugated product was seen only when all components of the ISG15 conjugating machinery were included in the transfection mix.

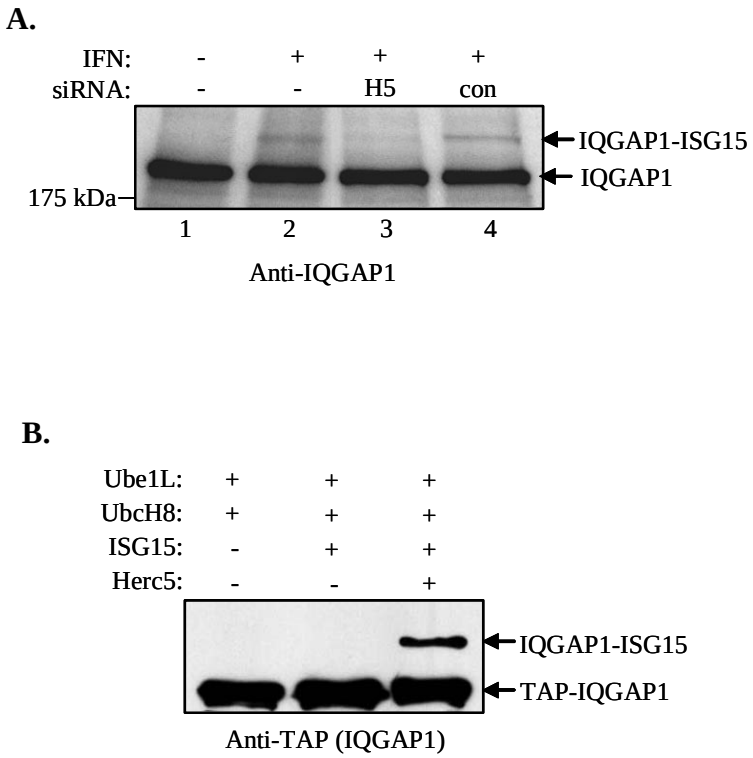


Figure 5.2 IQGAP1 is conjugated to ISG15 in vivo. A) HeLa cells were mock-transfected (lanes 1 and 2) or transfected with an siRNA against Herc5 (lane 3) or a control siRNA (lane 4). The cells were then treated with IFN-β for an additional 48hrs (lanes 2-4). Cell extracts were analyzed by immunoblotting with anti-IQGAP1 antibody. B) HeLa cells were transfected with plasmids expressing TAP-IQGAP1 and various combinations of Ube1L, UbcH8, ISG15 and Herc5. Cell extracts were immunoblotted with anti-TAP antibody.

siRNAs against IQGAP1 inhibit ISG15 conjugation

To determine whether IQGAP1 plays a role in promoting ISG15 conjugation, rather than simply being a substrate of ISG15 conjugation, two sets of siRNAs were designed to target IQGAP1 (IQ1A and IQ1B – See Materials and Methods). An immunoblot using anti-IQGAP1 antibody shows that at a final concentration of 100 nM, both sets of IQGAP1 siRNAs efficiently knock down IQGAP1 at the protein level (Fig 5.3 A), whereas the Herc5 siRNAs and a control siRNA do not affect it. A time-course was performed to determine the optimal time period of siRNA treatment. Figure 5.3B shows that cells harvested after 72 hours of siRNA treatment have lower levels of IQGAP1 as compared to cells that were harvested 24 hrs and 48 hrs after siRNA treatment. For all further experiments involving siRNAs, cells were harvested 72 hrs after transfection.

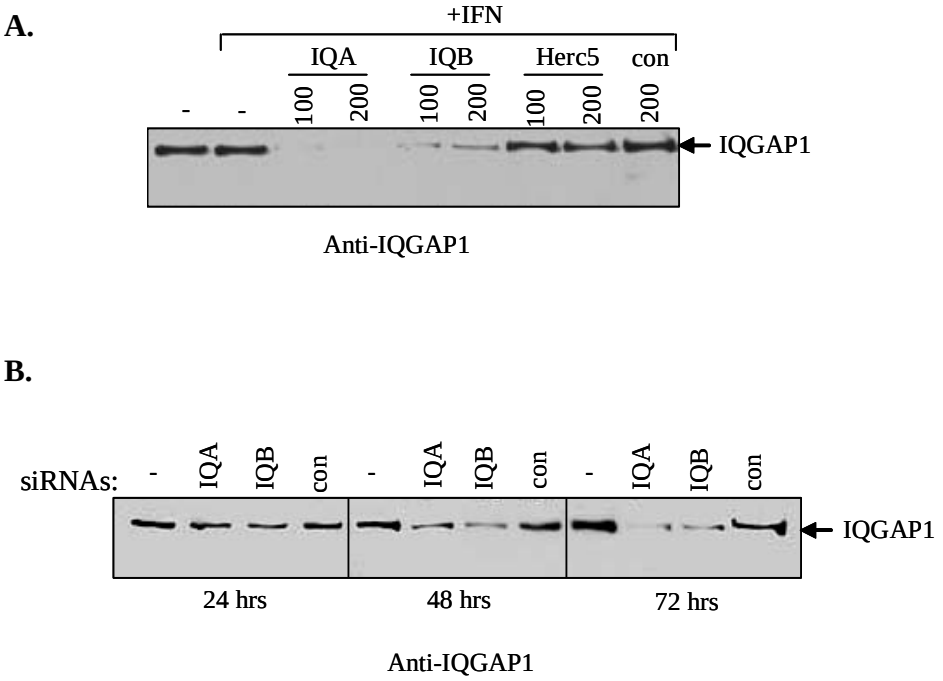


Figure 5.3 siRNAs against IQGAP1 effectively reduce the levels of IQGAP1 protein. A) HeLa cells were mock-transfected (lanes 1 and 2) or transfected with 100 nM and 200 nM of various siRNAs as indicated, and then treated with IFN-β for 48 hrs. Cell extracts were immunoblotted with anti-IQGAP1 antibody. B) A time-course to determine the optimal time period of siRNA treatment. HeLa cells were mock transfected or transfected with a control siRNA or two sets of siRNAs against IQGAP1. Cells were harvested after 24 hrs, 48 hrs or 72 hrs and extracts were immunoblotted with anti-IQGAP1 antibody.

In order to test whether IQGAP1 was involved in ISG15 conjugation, HeLa cells were transfected for 24 hrs with siRNAs to knock down IQGAP1 or Herc5 or a control gene, followed by IFN-β treatment for an additional 48 hrs. When immunoblots were probed with anti-ISG15 antibodies, an induction of high molecular weight ISG15 conjugates was seen in IFN-β-treated cells (Fig 5.4A lane 2). Cells in which IQGAP1 was knocked down (Fig 5.4A lanes 3-6), showed a reduced level of ISG15 conjugation compared to cells with no siRNAs (Fig 5.4A lane 2) or the control siRNA (Fig 5.4A lanes 7 and 8). The levels of ISG15 conjugation in IQGAP1 knock-down cells was higher than the cells in which Herc5 had been knocked down (Fig 5.4A lanes 9 and 10), and this is likely due to the inefficient IQGAP1 knock-down as compared to the Herc5 knock-down (compare Fig 5.3A and 3.1B).

We had previously shown that non-IFN-treated cells transfected with plasmids expressing ISG15, Ube1L, and UbcH8 showed a low level of ISG15 conjugation which was increased dramatically when Herc5 was included in the transfection (18). This reconstitution of ISG15 conjugation implied that any additional components required for ISG15 conjugation must be constitutively expressed. Figure 5.4B shows an accumulation of ISG15 conjugates with the triple (Ube1L, UbcH8 and ISG15) and quadruple (Ube1L, UbcH8 and ISG15 and Herc5) transfections. In this context, when cells were pre-treated with siRNAs against IQGAP1, they showed an overall decrease in ISG15 conjugation compared to control siRNAs (Fig 5.4B).

A.

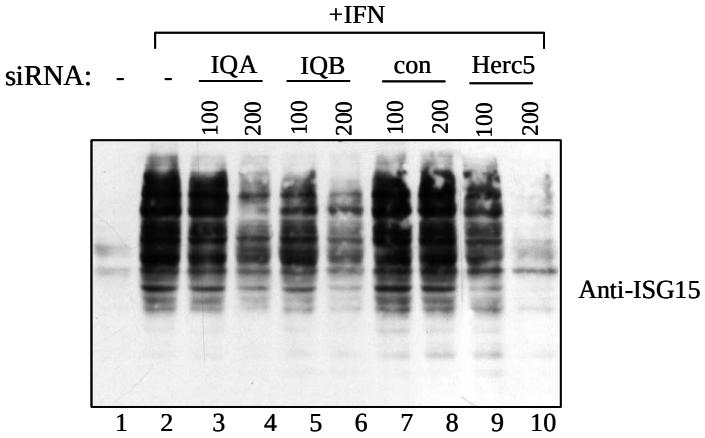
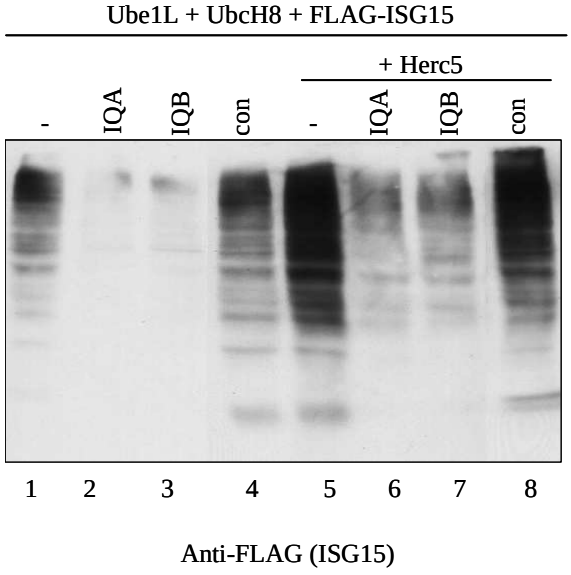


Figure 5.4 A siRNAs against IQGAP1 decrease ISG15 conjugation in IFN-treated cells. HeLa cells were mock transfected (lanes 1 and 2) or transfected for 24 hrs with siRNAs to knock down IQGAP1 (lanes 3-6) or Herc5 (lanes 9 and 10) or a control gene (lanes 7 and 8), followed by IFN-β treatment for an additional 48 hrs. Cell extracts were probed with anti-ISG15 antibody.

B.



5.4 B IQGAP1 is required for ISG15 conjugation in cells transfected with E1, E2 and ISG15. HeLa cells were mock transfected or transfected with IQGAP1 or control siRNAs. 24 hrs later, they were transfected with plasmids expressing Ube1L, UbcH8 and FLAG-ISG15 without (lanes 1-4) or with (lanes 5-8) Herc5. Cell extracts were transferred to a nitrocellulose membrane and probed with anti-FLAG antibody.

Having seen a global decrease in ISG15 conjugation in the presence of siRNAs against IQGAP1, we analyzed two individual ISG15 targets, Moesin and Thioredoxin Reductase 1 (TrxR1). The verification of these proteins as substrates of ISG15 conjugation has been reported previously (197). Moesin acts as an adaptor between plasma membrane and cytoskeletal proteins and was recently shown to have anti-retroviral activity (133). TrxR1 is a cellular enzyme (TrxR2 is its nuclear counterpart) that is involved in protecting cells against oxidative stress and is upregulated in a number of human cancers (132). When these substrates were transfected into non-IFN-treated HeLa cells in the presence of Ube1L, UbcH8, ISG15 and Herc5, we could detect their ISG15 conjugated forms. However, in the presence of siRNAs targeting IQGAP1, we did not detect the modified forms of these substrates, indicating that IQGAP1 is required for conjugation of ISG15 to these specific target proteins (Fig 5.5).

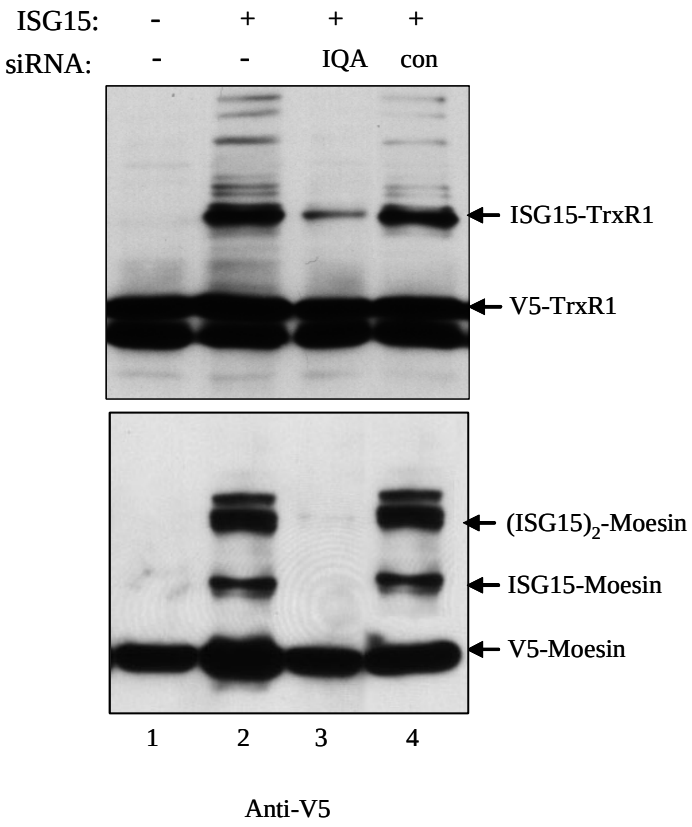


Figure 5.5 IQGAP1 is required for conjugation of ISG15 to TrxR1 and Moesin. HeLa cells were mock-transfected (lane 2) or transfected with IQ A (lane 3) or a control siRNA (lane 4). After 24 hrs they were transfected with plasmids expressing V5-TrxR1 (upper panel) or V5-Moesin (lower panel), along with Ube1L, UbcH8, ISG15 and Herc5. In lane 1 ISG15 was not included in the tranfection mix. Cell extracts were immunoblotted with anti-V5 antibody.

IQGAP1 binds Herc5 in vivo and in vitro

To determine whether IQGAP1 and Herc5 physically interact with each other, Herc5 was expressed as an N-terminally TAP-tagged protein in 293T cells and purified on IgG-sepharose beads. TAP-Herc4, TAP-Herc6 and TAP-E6AP were similarly expressed and purified as controls. IQGAP1 was expressed as a His-tagged protein and purified from insect cells (119). The TAP-tagged proteins immobilized on IgG-sepaharose beads were tested for binding to purified IQGAP1. Herc5, and to a lesser extent Herc6, bound to IQGAP1 (Fig 5.6A), whereas Herc4 and E6AP did not show binding above background. I also determined whether IQGAP1 acts as a scaffold to recruit UbcH8, the component upstream of Herc5 in the ISG15 cascade. TAP-UbcH8 was expressed and purified from 293T cells and was tested for interactions with IQGAP1. Figure 5.6B shows that UbcH8 did not bind IQGAP1.

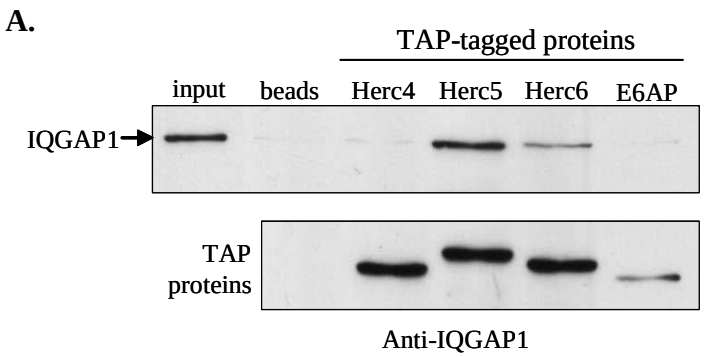


Figure 5.6 IQGAP1 binds Herc5 *in vitro.* Figure legend on next page.

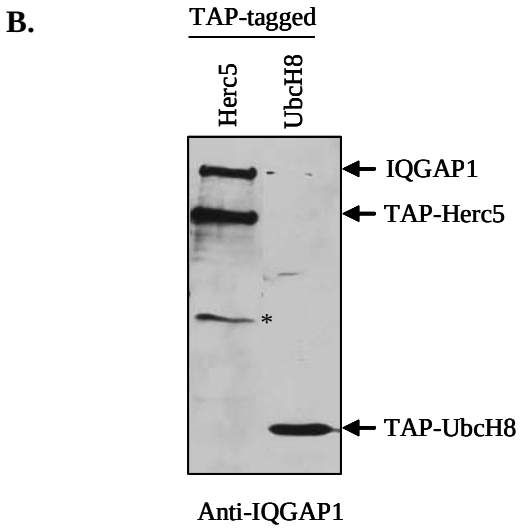


Figure 5.6 IQGAP1 binds Herc5 in vitro. A) TAP-tagged Herc4, Herc5, Herc6 and E6AP were purified from 293T cells, immobilized on Ig-G-sepharose beads and incubated with purified IQGAP1for 2hrs at 4 ^{o}C. The beads were subsequently washed, resuspended in 1X loading buffer and loaded on an SDS-PAGE. The gel was transferred to a nitrocellulose membrane and bound IQGAP1 was detected by western blotting. Lane 1 shows the input, which is 1/5 the amount used in the assays. Lane 2 indicates the amount of IQGAP1 bound to IgG sepharose beads only and is considered the background. The lower panel shows the TAP-tagged proteins. B) Same as A, except, TAP-tagged UbcH8 was tested for binding to IQGAP1. TAP-Herc5 was used as a positive control. A break down product of Herc5 is indicated by (*).

To define the domains of Herc5 that bind IQGAP1, a series of Herc5 truncations were expressed as TAP-fusions and purified from 293T cells (Fig 5.7). Residues 1-377 of Herc5 containing the RCC1-like repeats, were sufficient to bind IQGAP1 at a level comparable to full length Herc5 (Fig 5.8). A protein spanning residues 381-1024, that includes the putative substrate binding box (SBB) and the HECT domain, was unable to bind IQGAP1, as was the HECT domain alone (Fig 5.8). Therefore, the determinants of Herc5 required for binding IQGAP1 lie within the first 377 amino acids of the protein. This region of the protein consists of four tandem RCC repeats from residues 156-377, with a fifth RCC repeat with low match score (Pfam) between residues 129 and 153. No recognizable domains are present within the first 128 residues of Herc5.

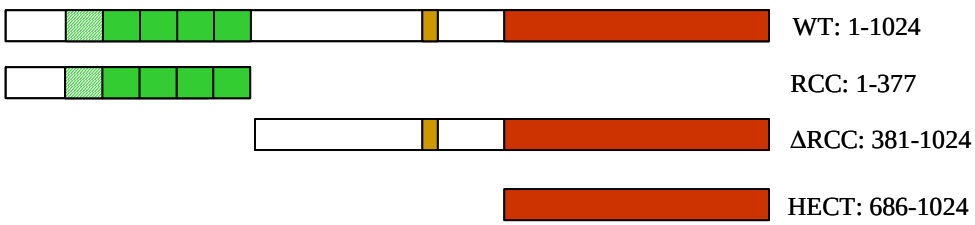


Figure 5.7 A schematic showing the truncation mutants of Herc5 tested for binding to IQAP1. The RLD, SBB and HECT domains are shown in green, brown and maroon, respectively.

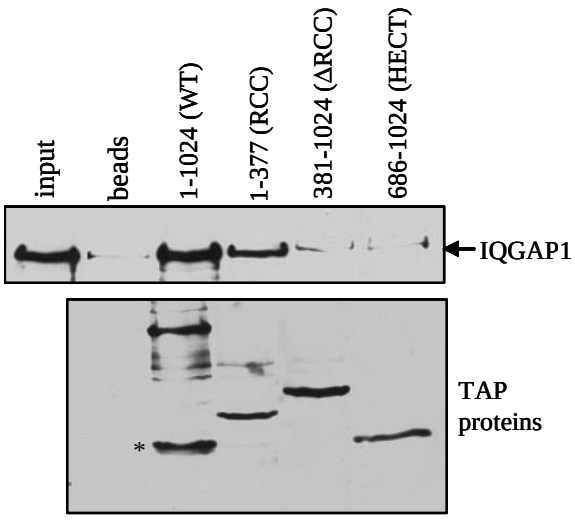


Figure 5.8 The N-terminal 377 amino acids of Herc5 are sufficient for binding IQGAP1. Wild type Herc5 and various mutants were expressed as TAP fusions in 293T cells. The TAP proteins were purified and immobilized on IgG-sepharose beads and incubated with purified IQGAP1for 2hrs at 4 ^{o}C. The beads were subsequently washed, resuspended in 1X loading buffer and loaded on an SDS-PAGE. The gel was transferred to a nitrocellulose membrane and bound IQGAP1 was detected by western blotting (upper panel). Lane 1 shows the input, which is 1/5 the amount used in the assays. Lane 2 indicates the amount of IQGAP1 bound to IgG sepharose beads only and is considered the background. The lower panel shows the TAP-tagged proteins. A break down product is indicated by (*).

To map the IQGAP1 domains essential for Herc5 binding, an *in vivo* binding assay was used. TAP-tagged truncation mutants of IQGAP1 (A-C, Fig 5.9) were co-expressed with Myc-tagged Herc5 in 293T cells. Extracts were bound to IgG sepharose beads and western blots were probed with anti-Myc antibody to detect bound Herc5. Figure 5.10 shows that a significant amount of Myc-Herc5 bound to IgG-sepharose beads in the absence of TAP-IQGAP1 protein, creating a high background for the binding assay. In spite of this, we could detect an increased amount of Myc-Herc5 bound to full-length TAP-IQGAP1 (Fig 5.10, lane 2 versus 3). The C-terminal 657 residues of IQGAP1 were sufficient to bind Herc5 (IQGAP1-A). This part of the protein contains two domains, the Ras-GAP related domain (GRD) and the Ras GAP C-terminus (RGCT), which have been previously shown to bind Rac1, Cdc42, β-catenin and E-cadherin (137). However, a larger construct spanning residues 525-1657 (IQGAP1-C), bound Myc Herc5 better than the one with the last 657 amino acids only. This indicates that there might be more than one determinant within IQGAP1 for binding Herc5. IQGAP1-C includes a WW domain and four IQ repeats besides the GRD and RGCT. WW domains are protein-protein interaction motifs with a known preference for PPXY sequences. An inspection of the Herc5 sequence did not reveal any PPXY motifs. The construct spanning the N-terminal 525 residues of the protein (IQGAP1-B) did not show any significant binding to Myc-Herc5. The results indicate that the C-terminal 657 amino acids of IQGAP1 mediate Herc5 interactions. In the future, both the domains comprising the C-terminus, that is, the GRD and RGCT, will have to be expressed separately to determine whether one of these binds Herc5 or the two of them in combination are required for Herc5 binding.

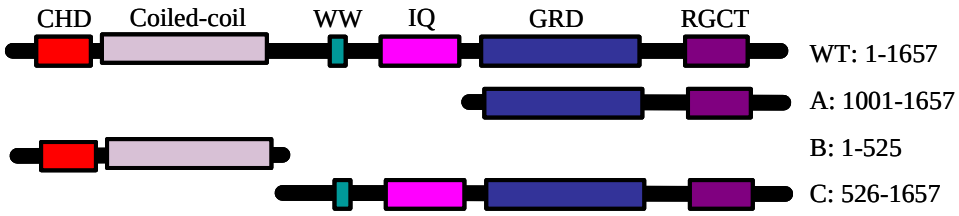


Figure 5.9 A schematic of IQGAP1 mutants used to test for binding to Herc5.

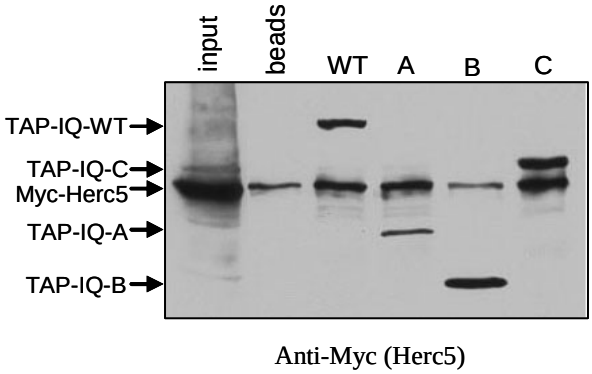


Figure 5.10 The C-terminal 657 residues of IQGAP1 are sufficient to bind Herc5 in vivo. TAP-tagged IQGAP1 truncation mutants (A-C) were co-expressed with Myc-tagged Herc5 in 293T cells. Extracts were bound to IgG sepharose beads for 2 hrs at 4 ^{o}C after which the beads were washed, and loaded on an SDS-PAGE. The gel was transferred to a nitrocellulose membrane and the blots were probed with anti-Myc antibody to detect bound Herc5. Lane 1 shows the input and lane 2 shows the Myc-Herc5 bound to beads only (background).

IQGAP1 affects activity of wild type Herc5 but not ΔRCC or ΔSBB mutants

As shown previously, Herc5 is able to conjugate ISG15 to itself, similar to other ubiquitin E3s that have the ability to ligate ubiquitin to themselves. This self-conjugation activity is absent in the C994A mutant of Herc5, which is a catalytically inactive enzyme. If IQGAP1 was functioning simply as an adaptor protein to recruit substrates to Herc5, knocking down IQGAP1 would not be expected to affect its ability to catalyze auto-conjugation. To determine whether IQGAP1 was required for the auto-conjugation function of Herc5, HeLa cells were treated with siRNAs against IQGAP1, and subsequently transfected with Ube1L, UbcH8, ISG15 and TAP-Herc5 expressing DNA. Fig 5.11 shows that there was less ISG15-modified Herc5 in cells where IQGAP1 was knocked down, whereas the control siRNA had no effect. This suggests that IQGAP1 is required for the catalytic activity of Herc5.

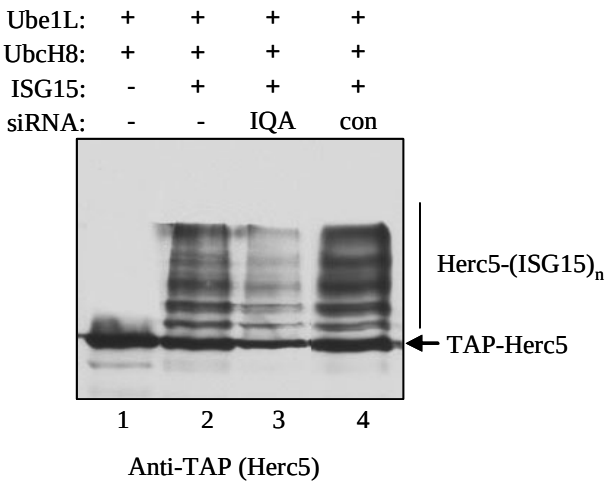
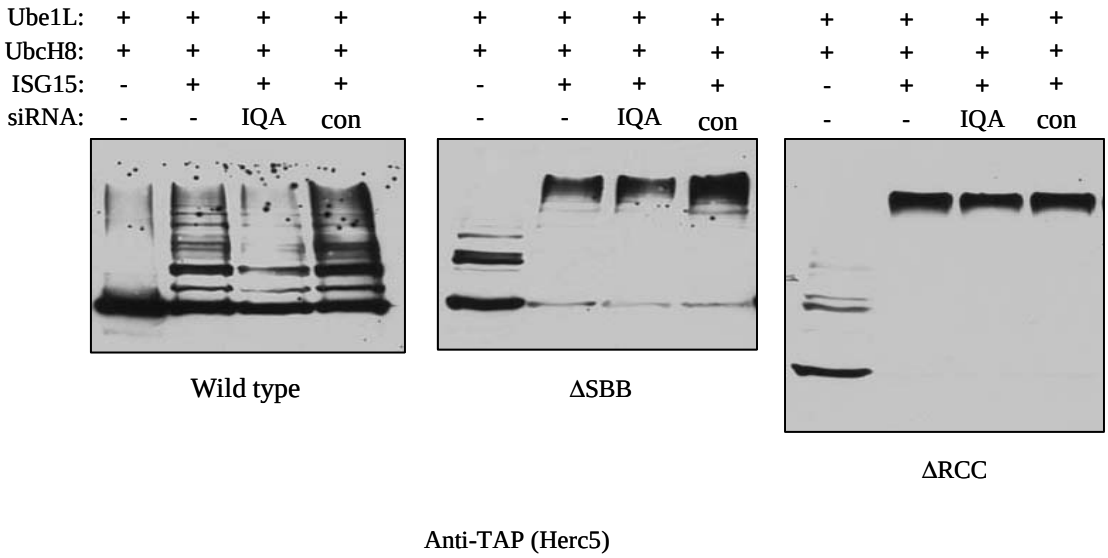


Figure 5.11 IQGAP1 is required for Herc5 auto-conjugation. HeLa cells were mock transfected (lane2) or transfected with IQA (lane 3) or a control siRNA (lane 4). After 24 hrs, they were transfected with plasmids expressing Ube1L, UbcH8, ISG15 and TAP-Herc5. Cells were harvested 48 hrs post-transfection and extracts were immunoblotted with anti-TAP antibody.

I also determined whether the activity of other Herc5 mutants is similarly affected by IQGAP1. As shown in Chapter 4, the ΔRCC1 and ΔSBB mutants retain the ability to auto-conjugate ISG15 and both the mutants were able to auto-conjugate ISG15 better than wild type Herc5. As with the wild type protein, anti-TAP western blots were used to detect auto-conjugation of Herc5 mutants. Fig. 5.12 shows some wild type protein remained unmodified whereas under similar conditions, both ΔRCC1 and ΔSBB mutants were completely conjugated and appeared as a smear of high molecular weight conjugates at the top of the gel. Unlike wild type Herc5, the auto-conjugation activity of these mutants remained unaltered in the absence of IQGAP1 (Fig. 5.12). These results suggest a model in which the wild type protein is in an inactive conformation and requires IQGAP1 for its activity. In contrast, the ΔRCC1 and ΔSBB mutants are constitutively active and hence are unaffected by the presence or absence of IQGAP1. The fact that both the mutants show increased activity suggests that the N-terminus of the protein might be interacting with the SBB to keep Herc5 in a closed, catalytically inactive conformation and deleting either of these domains relieves the inhibition. I speculate that the wild type protein might be activated by binding of IQGAP1 to the RCC repeat region.



5.12 IQGAP1 is not required for auto-conjugation of ΔRCC and ΔSBB mutants. HeLa cells were mock transfected or transfected with IQA siRNA or a control siRNA, as indicated. After 24 hrs, they were transfected with plasmids expressing Ube1L, UbcH8 and ISG15 along with wild type TAP-Herc5 (left panel), ΔSBB mutant (middle panel) or ΔRCC mutant (right panel). Cells were harvested 48 hrs post-transfection and extracts were immunoblotted with anti-TAP antibody.

5.3 Discussion

Ube1L and UbcH8 are the primary E1 and E2 enzymes for ISG15 respectively. My previous work showed that Herc5 is the major ligase for this Ubl. Here we identify another factor, IQGAP1, as being required for Herc5-dependent ISG15 conjugation.

IQGAP1 was identified in a proteomics study as one of many proteins modified by ISG15. My results confirmed this observation and further showed that the modification of IQGAP1 is Herc5-dependent, as is most, if not all, ISG15 conjugation. It is typical for enzymes of a Ubl pathway to be modified by their respective Ubls (21, 145), and IQGAP1 might be similarly conjugated simply based on its close association with Herc5. It is not known whether IQGAP1 modification is, in turn, required for its activity in the ISG15 conjugaiton pathway. Future experiments may identify the site(s) of ISG15 modification on IQGAP1 in order to determine whether modification at those sites is required for its function in the ISG15 system.

As mentioned in Chapter 3, an *in vitro* ISG15 conjugation assay was reconstituted using purified Ube1L, UbcH8, ISG15 and Herc5. Herc5 purified from insect cells did not form a thio-ester bond with ISG15, nor did it modify known, purified substrates. However, when transfected 293T cells were used as a source of Herc5, conjugation was detected. This might now be explained in light of IQGAP1. Here it has been shown that knocking down IQGAP1 inhibits the activity of Herc5, and hence the Herc5 purified from insect cells (which do not have an IQGAP1 homolog) would not be active without IQGAP1 in the reaction mix. However, in spite of including purified IQGAP1 in the *in*

vitro reaction assay I did not detect a Herc5~ISG15 thio-ester bond. This could mean that yet other factors required for ISG15 conjugation are missing.

ISG15 was shown to act as an antiviral molecule in mice infected with influenza virus and Sindbis virus and conjugation of ISG15 to substrates was important for this function (105). It might be predicted that a protein which boosts ISG15 conjugation might also have an anti-viral role. My data shows that IQGAP1 is needed for activating Herc5, which in turn is necessary for conjugating ISG15 to substrates and hence, IQGAP1 could be a potential anti-viral protein. So far, there have been no reports that tested this possibility. Mice lacking IQGAP1 have no obvious phenotypes other than a propensity for gastric hyperplasia (108), but no experiments were reported that would have specifically determined the response of these mice to viral infections. IQGAP1-/- mouse embryonic fibroblasts have been recently described (153), and these might be useful for testing whether IQGAP1 is involved in antiviral responses.

Binding studies show that IQGAP1 interacts with Herc6, but the binding is less as compared to that seen with Herc5. This is consistent with results showing Herc5 to be the major E3 for ISG15, with Herc6 playing a minor role. Herc4, which does not play any role in ISG15 conjugation, did not interact with IQGAP1. It is tempting to speculate that the interaction of Herc5 with IQGAP1 might be responsible for recruiting substrates to Herc5 to be ISG15 conjugated. IQGAP1 is a 189 kDa protein with multiple domains and it is plausible that different subsets of target proteins bind different domains. However, in preliminary experiments to pull down substrates from IFN-β-treated cell extract on TAP-IQGAP1-IgG-sepharose, no ISG15 conjugates were detected (data not shown).

My results suggest that IQGAP1 interacts with Herc5 and activates it. There have been other reports of IQGAP1 modulating the activities of its binding partners. For example, IQGAP1 inhibits the β-catenin-E-cadherin complex, leading to decreased cell-cell adhesion (103), whereas it enhances the co-transcriptional activation function of β-catenin in a calmodulin-dependent manner (11). IQGAP1 also alters the EGF-mediated activation of B-Raf, MEK and ERK2 proteins (153, 158, 159). Further, a recent report claimed that IQGAP1 decreased the amount of activated Rap1, which is in contrast to its effect on other small GTPases like Cdc42 and Rac1 (76). No enzymatic activity has so far been detected for IQGAP1 and in all the above examples it modulates the activity of its interacting partners by either displacing other proteins from a complex or recruiting activators/inhibitors. It is possible that in the case of Herc5 too, IQGAP1 recruits a protein which in turn activates the ligase.

The results so far, indicate a model in which Herc5 is in a closed conformation and requires IQGAP1 to activate it (Fig 5.13). I speculate that the N-terminus of Herc5 might bind to the SBB to keep the protein in a closed inactive state. When IQGAP1 binds the RCC1-like repeats, it disrupts the intramolecular interaction between the N-terminus and the SBB and the protein is activated. Additionally, IQGAP1 might also function by recruiting substrates to Herc5 by virtue of its modular structure. This would explain why the ΔRCC1 mutant, which does not interact with IQGAP1, could conjugate ISG15 to itself, but not to other substrates. The HECT E3 Smurf2 is similarly regulated by an auto-inhibitory mechanism (140). The intramolecular interaction between the C2 and HECT

domains inhibits Smurf2 activity. The inhibition is counteracted by an adaptor protein Smad7, which binds the HECT domain and activates the ligase activity. Future experiments in the lab will be aimed towards testing this model. I will investigate whether the N-terminus of Herc5 has an affinity for its C-terminus, as predicted by this model. The model also predicts that adding increasing amounts of the Herc5 N-terminal fragments should relieve the auto-inhibitory conformation by competing for binding to the SBB, and in that case, the protein should no longer be dependent on IQGAP1 for activation. Also, efforts will be made to reconstitute an *in vitro* assay, since biochemisty will greatly enhance our understanding of this system. Further, I cannot completely rule out the possibility that IQGAP1 might interact with other enzymes of the ISG15 pathway, although, so far I have no evidence that supports it. As mentioned in the introduction, IQGAP1 co-ordinates multi-protein complexes that are important for cell motility, cell-cell adhesion, and MAP kinase signal transduction. It might similarly function to increase the local concentrations of ISG15 enzymes and prevent ubiquitin enzymes like E6AP from interacting with the ISG15 pathway proteins *in vivo*.

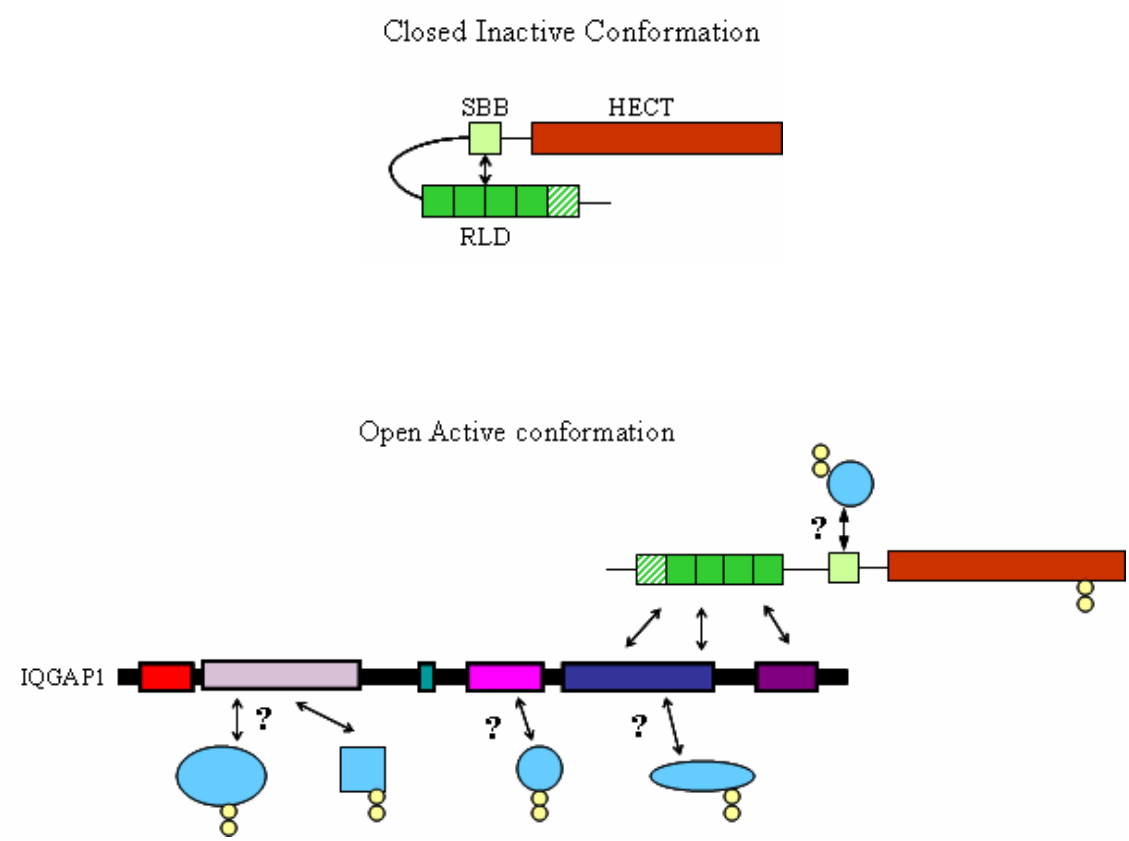


Figure 5.13 A model for the role of IQGAP1 in Herc5-mediated ISG15 conjugation. The N-terminus of Herc5 might bind to the SBB to keep the protein in a closed inactive state. When IQGAP1 binds the RCC1-like repeats, it disrupts the intramolecular interaction between the N-terminus and the SBB and the protein is activated. Additionally, IQGAP1 might also function by recruiting substrates to Herc5 by virtue of its modular structure.

CHAPTER 6: FUTURE DIRECTIONS

My work has shown that Herc5 is the major ligase for ISG15, an antiviral ubiquitin-like protein, and that IQGAP1 interacts with Herc5 and is required for Herc5 activity *in vivo*. I have identified two novel factors that play a role in ISG15 conjugation, and these findings add significantly to our knowledge of the ISG15 pathway.

One of the most important aspects of my work is that it will be useful for reconstituting an *in vitro* system for ISG15 conjugation. Previously, Ube1L and UbcH8 had been identified as the E1 and E2 enzymes, respectively, for ISG15. Also, a proteomics approach had identified more than 150 proteins as substrates of ISG15 conjugation. With the discovery of the E3, and its associated protein IQGAP1, we may have identified all the factors required for reconstitution of ISG15 conjugation. An *in vitro* assay will be an invaluable tool that will help us address many questions. It will be crucial for determining the biochemical consequences of ISG15 modification of target proteins. Modification of targets by other Ubls (e.g., Sumo) causes changes in localization, activity, or protein-protein interactions. ISG15 may be performing similar roles, although so far there is little evidence for any of the above. ISG15 modification of Ubc13 was reported to abrogate its ability to form a thioester bond with ubiquitin because the ISG15 modification site was just 5 residues from the active site cysteine and stearic hindrance prevented the interaction with ubiquitin (170, 198). Although the protein phosphatase 2Cbeta (PP2Cbeta), which is involved in NFκB pathway, was shown to have

a decreased activity when it was conjugated to ISG15, the effect was very subtle (169). 4EHP, an mRNA cap-binding protein and a suppressor of translation, was reported to be modified by ISG15 via the RING-type ligase HHARI and this increased its cap-binding affinity (142) . However, the data suggests that the increase in cap-binding affinity was not significant. None of these reports gave a convincing demonstration of the effect of ISG15 conjugation. An *in vitro* conjugation system for ISG15 would be useful for determining the biochemical effects of ISG15 on specific activities of target proteins. We could isolate the modified product and look for changes in its ability to interact with known binding partners. Further, such a system would be essential to understand the biochemistry involved in Herc5-dependent conjugation. It would allow us to isolate any further factors that might be required for ISG15 conjugation and/or recruitment of specific target proteins to the Herc5/IQGAP1 complex. Mutants of Ube1L, UbcH8, Herc5 and ISG15 could be tested for activity without the complications involved in *in vivo* assays. The effect of other factors such as IQGAP1 would also be more clearly understood using biochemistry. The *in vitro* system described in Chapter 3 is a good starting point for establishing assays for Herc5 activity. Although those assays are crude and use whole cell extract, they are the first direct evidence of Herc5 activity. In the future, that assay will have to be refined and used with purified Herc5 and IQGAP1.

An important aspect that needs to be studied is the identification of sites of ISG15 modification on substrates. Although we now have a list of more than 150 substrates modified by ISG15, the sites of ISG15 attachment to only Ubc13, EFP and PP2Cbeta are known (169, 198, 199). As more data about the conjugation sites accumulates, it might

yield a consensus structure/motif that is recognized by Herc5. In addition, we will be able to mutate the sites and transfect the mutant proteins into cells to check for a change in localization and/or function. This is especially important for known antiviral proteins, including p56 and nine other antiviral proteins that were identified as substrates for ISG15 conjugation. In the case of IQGAP1, identification of its site of modification will allow us to determine whether ISG15 conjugation is required for its Herc5-related function. It will also be important to ascertain the fidelity of any *in vitro* conjugation system and one of the ways to do that would be to check whether the same sites are being modified on a substrate *in vitro*, as they are *in vivo*. Further, identifying sites of ISG15 modification will allow us to determine whether poly-ISG15 chains are formed on target proteins. Most substrates modified by ISG15 show a single conjugate. However, some like p56 and moesin, exhibit multiple conjugates. Recently, SUMO was shown to form short chains (99) and it is possible that ISG15 forms chains, given that the K-48 residue on ubiquitin is conserved in the C-terminal domain of ISG15 (K-129).

A significant aspect of my work is the discovery that a HECT E3 was shown to function with a Ubl other than ubiquitin. While the RING E3s function with both ubiquitin and some Ubls (128, 186), HECT domain proteins were known to act as E3s for ubiquitin only. This new finding further broadens the spectrum of biological processes that HECT proteins participate in. My preliminary results with the active site mutant (C994A) and the ΔF mutant suggest that the mechanism of ubiquitin and ISG15 conjugation are identical. So far, we know that HECT domains have the inherent structure to function with ISG15 and this was shown by *in vitro* experiments with E6AP

and Rsp5 (196).What we do not know is whether Herc5 can function with ubiquitin or whether it is exclusively devoted to ISG15. It is essential to make active Herc5 *in vitro*, so that we can test whether it functions with ubiquitin. If Herc5 does not form a thioester with ubiquitin *in vitro*, then we can investigate the determinants within Herc5 that prevent it from functioning with ubiquitin.

Another interesting result of my work was that Herc6 was shown to function with ISG15, albeit not as efficiently as Herc5. It was not clear why the chimera Herc5/6, which had the Herc6 HECT domain and the rest of the protein from Herc5, did not support conjugation of ISG15 to substrates. There might be unique features of the N-lobe or C-lobe of Herc5 and maybe making chimeras in this region of the protein might be useful. As discussed in Chapter 4, I suggest that since Herc5 is absent in rodents, Herc6 might be the major ligase for ISG15 in these organisms. This possibility needs to be tested. Herc6 can be knocked down in interferon-treated mouse cells to determine whether it has an effect on substrate conjugation. If Herc6 is important for ISG15 conjugation in mice, knockout mice that lack the gene for Herc6 can be created. This would provide an animal model to test the role of ISG15 ligases and might provide us with some new insights into non-ISG15-related roles for Herc6 and Herc5. Such a mouse model would resolve the confusion in the ISG15 field about the role of the other ligase TRIM25/EFP. Although I did not see an overall effect on ISG15 conjugates when TRIM25/EfP was knocked down, it has been postulated to function as an ISG15 ligase for the negative cell cycle regulator 14-3-3σ (200). The finding that it conjugates ISG15 to the same substrate (14-3-3σ) which it ubiquitinates, could be because in interferon-

treated cells, the high levels of UbcH8, ISG15 and Efp/TRIM25, might force the ligase to react with ISG15. Also, the levels of Efp auto-conjugated to ISG15, were much lower than the levels of ubiquitin-conjugated Efp (134), suggesting that it functions more efficiently with ubiquitin. Finally, a recent report by Gack et al showed that Efp/TRIM25 was essential for RIG-I mediated anti-viral activity (35). Efp/TRIM25 mediates robust ubiquitination of the caspase recruitment domains (CARDS) of RIG-I, which enhances RIG-I downstream signaling. This role of Efp/TRIM25 might explain why a ubiquitin ligase is upregulated in the presence of interferons. A Herc6 knockout mouse would be very useful to clarify whether there are any redundant ISG15 ligases. If no ISG15 conjugates are detected in such a mouse, it would conclusively tell us that Herc6 (and Herc5 in humans) is the only ligase for ISG15.

Besides Herc5, I also identified a non-interferon induced protein, IQGAP1, which is essential for ISG15 conjugation. My results suggest that IQGAP1 might be functioning in the ISG15 pathway by binding the N-terminus of Herc5 and facilitating its activation. Further experiments will need to be done to test the model. For example, we need to determine whether the N-terminus of Herc5 interacts with the SBB. Also, given the modular nature of IQGAP1, it might function as an adaptor to recruit substrates or it might act as a scaffold to recruit the enzymes of the ISG15 pathway. The IQGAP1-/- cell line (153) is a useful tool and can be used in future experiments to test various mutants of IQGAP1 and their ability to support ISG15 conjugation.

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